## THE JOURNAL OF EXPERIMENTAL MEDICINE



## THE JOURNAL

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EDITED BY

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VOLUME SIXTIETH WITH FORTY-EIGHT PLATES AND FIFTY-FIVE FIGURES IN THE TEXT



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## THE PRODUCTION OF STREPTOCOCCUS HEMOLYTICUS BACTEREMIA IN NON-SPECIFICALLY SENSITIZED

By CASPAR G. BURN, M.D., CAROLINE A. CHANDLER, M.D.,

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(Received for publication, March 15, 1934)

During the course of studies concerning the relation of the hypersensitive state to localization of bacteria, Weisberger (1) observed that there was a prolongation of bacteremia in rabbits previously sensitized to normal horse serum. Rabbits hypersensitive to horse serum were inoculated simultaneously with a sublethal quantity of Streplococcus viridans following which blood cultures were positive for many hours, in some instances as long as 72 hours. On the other hand, in non-sensitized animals that received a similar inoculation of horse serum and bacteria, the organisms disappeared in 1½ to 2 hours after inoculation.

Due to the frequency of a sensitized condition of individuals to horse serum and the fact that antisera prepared in the horse are employed as therapeutic agents in certain infectious diseases, is it not possible that a similar phenomenon may be the basis of the change observed frequently in the clinical course of these diseases following shortly after the injection of antiserum?

As an approach to study of the subject, experiments were designed to observe the behavior of non-specific and specific sensitized animals with respect to bacteremia following simultaneous inoculation of antigen and a scarlet fever strain of hemolytic streptococci. It is the purpose of this communication to present the results of experiments in which antisera prepared in the horse were employed as antigens to inject simultaneously with the bacteria into rabbits sensitized to normal

A 20 to 22 hour culture of a hemolytic streptococcus recovered from a case of scarlet fever (Strain 273) was used throughout these experiments. Studies of the

rate of growth of this streptococcus in broth showed the maximum period of active growth to occur between the 8th and 10th hours. Depending on the quantity of culture desired, 1 or 2 cc. of the broth culture was centrifuged, the supernatant liquid decanted and the bacterial sediment resuspended in 10 cc. of sterile saline. Exactly 1 cc. of the suspension was injected into the ear veins of normal and sensitized animals from the same syringe. In order to eliminate factors such as change in virulence<sup>1</sup> of the organism, difference in the quantity of bacteria and variations in volume injected, the control and the sensitized animals were injected as nearly simultaneously as possible. The quantity of suspension injected was kept constant at 1 cc. for the entire series. In order to guard against change in bacterial growth upon standing, the whole procedure was so timed that the interval between the removal of the broth culture from the incubator and the injection of the suspension was never greater than 20 minutes.

All the rabbits were obtained from the same source, and in each pair of animals run simultaneously, the weight variation was never greater than 2/5 kilo. Those animals selected for sensitization were given three intraperitoneal injections of normal horse serum at 4 day intervals. After a period of 18 to 21 days the animals were skin tested for sensitivity by intracutaneous injection of normal horse serum in dilutions of 1:5 and 1:10. The skin reactions were controlled by intracutaneous injection of normal saline. Those animals giving strongly positive skin reactions to both dilutions as evidenced by local erythema and edema were taken for experimental use. Animals giving equivocal or negative skin reactions were discarded with one exception. This animal was used as the control as shown in Table I. The kinds of sera used in this study and their source are as follows: (1) normal horse serum, (2) scarlet fever antitoxin and (3) diphtheria antitoxin (Eli Lilly and Company—without preservative).

From 7 to 9 days after operation, the animals were sacrificed with ether and necropsies performed. Cultures were made from the liver, the heart's blood and the spleen. In certain of the animals, histological sections of the tissues were made and routine hematoxylin-eosin and Gram stains were prepared.

A typical protocol of an experiment is given.

August 31, 11.30 a.m. Rabbit 1, weighing 1600 gm.; ear vein shaved. Rabbit 2, weighing 2000 gm.; shaved, prepared and the jugular vein exposed under ether anesthesia. 12.55 p.m. Operation complete. 1.00 p.m. Culture removed from incubator. 2 cc. transferred to sterile centrifuge tube and centrifuged 10 minutes. 1 cc. transferred from same pipette to 9 cc. of sterile saline in first tube of dilution series. Dilutions run out to 10<sup>-7</sup>. 1.00 p.m. Plate poured from each dilution.

<sup>&</sup>lt;sup>1</sup> Virulence.—1 cc. of a 24 hour broth culture killed an albino mouse in a dilution of 10<sup>-1</sup> (500 million per cc.) within 17 hours. With higher dilutions all the mice survived and when autopsied on the 7th day, gave uniformly negative cultures. Virulence as tested by this method was the same at the end as at the beginning of the experimental period.

Dose to be calculated from these counts. 1.12 p.m. Supernatant liquid decanted and organisms resuspended in 10 cc. of saline. 1.15 p.m. Rabbit 1 injected in ear vein with 1 cc. of saline suspension. 1.17 p.m. Rabbit 2 injected in ear vein with 1 cc. of saline suspension (from same syringe) and a few seconds later injected in same ear vein with 1 cc. of 1:10 normal horse serum. No symptoms of shock noted. 1.30 p.m. Blood drawn from jugular of Rabbit 2. A few drops transferred to broth. 1 cc. put in plate and plate poured.

Following this, blood was drawn from the jugular vein in the same manner at 30 minutes, then every hour for the first 3 hours and finally at 6, 9, 12 and 24 hours. The incisions were closed with a few sutures and the animals returned to their cages after the 3 hour period and after every subsequent bleeding. hour period, blood cultures were taken by means of cardiac puncture.

TABLE I

		TABLE I	Pane	ture.
Series I	Amount of horse seru	Quantity of organ- isms inoculated per	Result of	blood cultures
1 (n)		cc.	Maximum No. of colonies of blood per cc.	
2 (s) 3 (n)	1 cc. of 1:10	1.6 1.6	22	hrs.
4 (s) 5 (n)	1 cc. of 1:10	1.6 1.6	3 <u>4</u> 20	3 9
6 (s)	1 cc. of 1:10	6.0	26	26 80
7 (n) 8 (s)	1 cc. of 1:10	0.1	75	7 22
n, normal; s, sen	sitized.	0.1	15	6 10
CO.				-

The results observed in four non-sensitized and four sensitized rabbits in which bacteremias were studied are presented in Table I.

Blood cultures of the non-specifically sensitized animals remained positive for a longer period than those of the non-sensitized group. The periods of prolongation varied from 4 to 15 hours, but seemed to bear no relation to the initial quantity of organisms injected. Rabbit 3 had received sensitizing injections of horse serum but failed to give a positive skin reaction. Although the duration of the positive blood cultures in this animal was strikingly longer than in other controls,

the sensitized rabbit that received the horse serum and organisms showed positive cultures for a significantly longer time. Whether those animals that fail to show a skin sensitivity react differently from normal animals remains for further investigation. However, these experiments indicate that in rabbits that are non-specifically sensitized there is a tendency for prolongation of the duration of *Streptococcus hemolyticus* in the circulation when horse serum is inoculated simultaneously with the suspension of bacteria.

In order to study the effects of normal horse serum in altering the response of a non-sensitized rabbit to the inoculation of *Streptococcus hemolyticus*, two rabbits were inoculated simultaneously with normal

		Quantity of organisms inoculated per cc.	Result of blood cultures	
Series II	Amount of horse serum		Maximum No. of colonies of blood per cc.	Duration of cul- tures
		millions		hrs.
17 (n)	-	0.5	59	6
18 (n)	1 cc. of 1:10	0.5	26	6
19 (n) 20 (n)	1 cc. of 1:10	7.0 7.0	400 190	72 <del>+</del> 12

TABLE II

serum and a known suspension of bacteria. The results of this experiment are shown in Table II.

Simultaneous injections of normal horse serum and bacteria do not prolong the bacteremia in non-sensitized animals. When the quantity of the initial inoculation approaches the maximum limit or the lethal dose, the variations in duration of positive blood cultures is more evident. This fact emphasizes the necessity for use of quantitative methods in such a study.

The next group of animals includes studies in which scarlet fever and diphtheria antitoxins in horse serum are employed instead of normal horse serum. Both the non-sensitized and sensitized animals received similar injections of serum and organisms, as shown in Table III.

Table IV shows the rate of disappearance of the organism from the blood stream in one of these experiments.

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				SHURN	
		TABLE III			5
Series III	Amount of strepto- coccus antitoxin	Quantity of organisms inoculate in			
		isms inoculated per		f blood cultures	=
9 (n)	1 cc. of 1:10	millions	Maximum No. c colonies per cc.	Duration of posi- tive cultures	-
10 (s)	1 cc. of 1:10	1.0		hrs.	
11 (n) 12 (s)	1 cc. of 1:1	1.0	11 94	6	
13 (n)	1 cc. of 1:1	3.6 3.6	22	10	
14 (s)	1 cc. of 1:1	- 1	620	9	
	1 cc. of 1:1	7.0	34	12	
			132	48 48	
	T1	D=-		-	

			1 48
	TABLE IV		
Time after inoculation			_
		Colonies per cc.	
	3:	Series III	
min.	No. 9		_
15			No. 10
30	2		
hrs.	Õ	- 1	
1	Ū	- 1	16
2	2	- 1	6
3	11	- 1	
6	6	1	10
10	1	1	19
12	0	1	94
24	0	1	68
48	0	1	1
	0	1	0
		1	o
			Õ
	TABLE V		
Series IV	THE V		

٠.		TABLE V		
Series IV	Amount of diplether antitoxin			
	antitoxin	Quantity of organ-		
		isms inoculated per cc.		lood cultures
15 (n)				
16 (s)	1 cc. of 1:1	Fillions	colonies bet co-	Duration of posi- tive cultures
	1 cc. of 1:1	0.4		Cultures
		0.4	34	hes.
			263	24
				24

Although the difference in the prolongation of the bacteremia in the sensitized animals from the non-sensitized animals is only 0 to 4 hours in these experiments, there is, nevertheless, a definite increase in the actual number of organisms present in the blood cultures of the sensitized animals.

When diphtheria antitoxin horse serum is employed, similar results are obtained, as shown in Table V.

No evidences of gross anatomical change appeared in any of the animals. The only significant microscopic changes in the viscera were found in the kidneys where proliferation of fibroblasts, infiltration of small round cells, a few capsular adhesions and areas of hemorrhage were more marked in the sensitized animals. Gram stains of the tissues were uniformly negative for bacteria, which was consistent with the negative cultural findings obtained at necropsy.

## DISCUSSION

The results of our experiments present evidence in support of Weisberger's conclusions; namely, that a prolonged bacteremia occurs in the hypersensitive animal, the minimal duration being 9 hours as compared with 3 hours in the non-sensitized control animal, although the actual maximum and minimum colony counts in these animals exhibit no significant differences. Also, these results agree with those of Clawson (2) who has shown that streptococci are removed less rapidly from the blood stream of specifically sensitized rabbits than from the blood stream of normal rabbits within an interval of 15 minutes. ing the literature further concerning studies on experimental bacteremia in animals, it is surprising that few investigators apparently have had their interests engaged by the study of bacteremia in relationship to sensitization. In fact, the only other report bearing on this subject is that of Boone, Chase and Brink (3) who found that large numbers of B. prodigiosus are absorbed from the intestinal tract of dogs during an acute anaphylactic shock.

In our experiments we have extended the observations on experimental bacteremia to the study of the influence of antisera employed as antigens instead of plain horse serum. As shown by the data presented the actual increase in the number of bacteria for the periods studied is significantly greater in the sensitized animal, yet the duration

of the bacteremia is approximately the same as is observed in the normal animal, in contrast to the prolonged bacteremia of the sensitized 7 animal shocked by means of normal horse serum. Since it is generally believed that bacteria are in large part removed from the blood by the cells of the reticulo-endothelial system in the liver and spleen of animals, it is suggested that the sensitization process alters this attribute of these cells so that they become inefficient in removing the bacteria

However, in the experiments we have described it is only during the interval of the first few hours that greater bacteremia is observed in the animals shocked with antisera. Therefore, if it be assumed that the sensitization process affects the cells in the manner just stated, it is necessary to assume further that there is a differential action between normal horse serum and antisera. As a matter of fact, the differences in the reaction of animals sensitized to horse serum when shocked with antisera contrasted with plain horse serum have never been studied in detail. Further analyses of this phenomenon are in progress at the present time.

Rabbits sensitized to horse serum developed a bacteremia of 9 to 12 hours' duration when they were inoculated simultaneously with normal horse serum and a strain of Streptococcus hemolyticus, while the bacteria could only be isolated from the blood stream of non-sensitized animals within the first 3 hours after inoculation. On the other hand, when antisera are employed as the antigen for shocking the sensitized rabbits, there is a significant increase in the number of bacteria in the blood stream in contrast to the control animals, but there is no evidence of a prolongation of the bacteremia.

- 1. Weisberger, D., Proc. Soc. Exp. Biol. and Med., 1932, 29, 445. 2. Clawson, B. J., J. Infect. Dis., 1933, 53, 157.
- 3. Boon, T. H., Chase, E. M., and Brink, H. E., Proc. Soc. Exp. Biol. and Med.,



# STUDIES ON HOST FACTORS IN PNEUMOCOCCUS

I. CERTAIN FACTORS INVOLVED IN THE CURATIVE ACTION OF SPECIFIC ANTIPNEUMOCOCCUS SERUM IN TYPE I PNEUMOCOCCUS DERMAL INFECTION IN RABBITS

By KENNETH GOODNER, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research) (Received for publication, March 14, 1934)

There is a growing impression among many investigators in the field of immunology that the protective or curative action of any antibacterial serum, such as antipneumococcus serum, is definitely conditioned by factors intrinsic to the animal body. For several decades the emphasis in immunology has been placed on the highly specific elements involved in resistance to infection, with the result that the physiological factors which make possible the proper functioning of these specific elements have been largely neglected.

The antibody content of antipneumococcus serum can be accurately estimated by in vitro methods such as that of Heidelberger, Sia, and Kendall (1), but the determined titers are frequently not quantitatively reflected in the results obtained by protective or therapeutic tests in mice or rabbits. There remains, of course, the possibility that the antiserum contains important, but as yet unrecognized elements, other than the anticarbohydrate antibody. On the other hand, it is a common observation that these in vivo methods of evaluation give confusing results because of a lack of sharpness as to end-point. Thus in any series of apparently similar animals treated in exactly the same way some individuals may die whereas others survive. Investigators in this field have been much influenced by the experience obtained in the use of the neutralization method in evaluating antitoxic serum, the efficacy of any lot of serum being determined by the amount required to save a guinea pig from death following the administration of a given amount of toxin. Experience has shown that, in general, there is a definite ratio between the amount of toxin and the protective dose of the serum. In the matter of antipneumococcus serum every effort has been made to show that a similar ratio exists between the amount of serum and the number of infecting organisms, but in general the work in animals has not demonstrated any accurate or consistent

In another type of experiment it has been shown that, in rabbits immunized with heat-killed pneumococci, the degree of resistance to subsequent infection was not necessarily correlated with the specific antibody titer in the serum of the individual animal (2).

It is obvious that these facts can be variously explained, but the most rational assumption is that some individual host factors may decisively modify or qualify the function of the highly specific antibody. In other words, the result in any individual animal must depend roughly on algebraic sum of all specific and constitutional factors. This problem has been approached from many sides, most successfully perhaps by the genetic studies of Webster (3), but there still remains the probability that individual animals differ other than in the genetic characters. Some workers have suggested the importance of so called physiological maturation, others have mentioned the state of physiological reactivity, but the problem has remained unsettled because of the difficulties of devising a suitable experimental approach.

In order to acquire some knowledge of host factors we have employed several types of experimentation each of which involved the pneumococcus as the infecting organism. The first experiments to be presented deal exclusively with Type I pneumococcus infections in rabbits in which antipneumococcus serum (Type I) has been used as a specific curative or protective agent. The general plan has been to arrange controllable conditions so that the situation presented is near a threshold; the ideal experiment being one in which the number of infecting organisms and the amount of specific antiserum are constant in a large number of animals and yet the balance between them so sharply determined as to permit the survival of certain individuals and the death of others. Then by analysis of other individual variables, both physiological and immunological, one can determine if the result is correlated therewith. This ideal type of experiment has not been entirely achieved in the course of the present work, but the results of the experiments tend to be mutually supportive and furnish evidence of the existence and nature of certain host factors.

The intradermal injection in rabbits of Type I pneumococci leads to the development of a characteristic lesion in the skin and to a definite symptom-complex marked by a high fever and accompanied by an invasion of the microorganisms into the blood stream (4). With this type of infection the result is fatal in the great majority of instances if no curative measures are employed. When, however, type-specific antipneumococcus serum is injected intravenously in large amounts the disease process may be abruptly terminated as judged by a fall in temperature, by the disappearance of pneumococci from the blood stream and from the local lesion, and by the healing of the local lesion. In the earlier work this prompt alleviation of symptoms was emphasized, but Sabin (5), who used survival and death as criteria, called attention to the fact that with certain small amounts of the specific serum some animals died whereas others survived. This quantity of serum was designated the "subeffective amount" in contrast to the "effective amount," which is the amount required to bring about prompt recovery. The subeffective amount of specific serum has no relationship to prompt termination of the infectious process but concerns only the ultimate survival or death of the animal (6). In almost every instance in which the administration of the subeffective amount of serum leads eventually to survival, the effect seems to be that of providing conditions which prolong the life of the animal until its own immunity mechanism can be developed and the disease thus autoterminated. The use of subeffective amounts of specific serum therefore provides experimental conditions favorable for the study of certain factors which might influence the outcome of the infection.

## EXPERIMENTAL

The general technical procedures are those described in a previous paper on the dermal pneumococcus infection in rabbits (4).

Culture.—Pneumococcus Type I, original Neufeld strain; virulence such that 0.000,000,01 cc. produces a fatal infection in rabbits following intradermal inoculation.

Infection.—Healthy male rabbits weighing from 1,600 to 2,500 gm. were used. Animals were injected intradermally at a site midway on the flank area with 0.2 cc. of a 1-200 dilution of an 18 hour blood broth culture.

Serum Treatment.—Each rabbit received a single intravenous injection of Type I antipneumococcus serum 24 hours after infective inoculation.

Blood Cultures.—The number of organisms per cubic centimeter of blood was determined by a procedure previously described in detail (4). In brief, the method consists in plating 0.4 cc. of blood withdrawn from the marginal ear vein with an accurately graduated syringe.

The general characteristics of the pneumococcus dermal infection in rabbits have been so frequently and extensively described that they need not be here repeated (4).

## Survival Rate after Treatment with Various Amounts of Specific Antiscrum

In order to determine the subeffective ranges of certain lots of Type I antipneumococcus sera four series of rabbits were treated with

Ι

varying amounts under the conditions described above. The results of one typical series are shown in Table I.

The 78 rabbits included in this series received amounts of the specific antiserum (Lot 1) ranging from 1 to 16 cc. Of the 3 animals which received 1 cc. each, none survived, while on the other extreme the 4 rabbits which received 16 cc. all survived the infection. Of 42 rabbits which were given 8 cc., 20 or 48 per cent survived. 36 per cent of those which received 4 cc. recovered while only 25 per cent of the animals treated with 2 cc. of serum survived.

This experiment demonstrates that between certain limits of serum dosage the administration of the specific therapeutic agent results in the survival of certain animals although others succumb. It is per-

TABLE I

Survival Rate after Treatment with Various Amounts of Specific Antiscrum

Each rabbit infected intradermally with Pneumococcus Type I. Designated amount of antipneumococcus serum (Lot 1) administered intravenously 24 hour after infective inoculation.

Group	Amount of serum given to each animal in group	No. of animals in group	Rabbits surviving		
			No.	Per cent	
	cc.				
A	16	4	4	100	
В	8	42	20	48	
С	4	22	8	36	
D	2	8	2	25	
$\mathbf{E}$	1	3	0	0	

haps noteworthy that the fourfold increase in amount of serum from 2 to 8 cc. resulted in only a twofold increase in the survival rate. In other series the quantitative effect of serum dosage in this subeffective range has been less marked.

Previous experiments have shown that in any series of rabbits, even though each individual has received the same infective inoculum, the experimental disease may progress more rapidly in some animals than in others. This is clearly demonstrated by differences in the rate of blood invasion and, in untreated animals, by differences in the time of death. The first analyses to be presented deal with the severity of the infection as a factor in the determination of the outcome.

Results of Serum Therapy in Relation to the Severity of the Infection

In order to determine if the severity of the infection might be correlated with the results in terms of survival and death an analysis was made of the data obtained from four series of animals similar to that shown in Table I. If the severity of the infection as indicated by the number of viable pneumococci per cubic centimeter of circulating blood were the only factor which determined the result it might be expected that with a given serum dosage all animals with severe infections would die whereas those with mild infections would survive, and further, that increasing amounts of serum would compensate for increasingly severe infections. The analyses, too detailed to reproduce in full here, showed that none of these assumptions was entirely true. Thus among rabbits treated with the same amounts: of the same serum some animals with bacteriemias of low degree died whereas others with extremely high bacteriemias survived. A division of the animals into groups showed, however, that the larger percentage of animals with low bacteriemias responded favorably after the injection of a subeffective amount of serum, while the larger proportion of those with high bacteriemias eventually died. Two analyses of this type are shown in Table II. For the purpose of correlation two end-points have been used. The first is that of death, 14 days being reckoned as the limit of experimental observation. The second endpoint is that of death within 4 days. This time period was arbitrarily chosen as including the larger number of the animals which died and yet sharply separating others in which the result was much less critical. Sabin (5) has already described the phenomenon of delayed death, and the present work confirms his observation that these delayed deaths are frequently associated with negative blood cultures and hence decidedly of a class differing from that which includes the earlier deaths for the latter are invariably associated with overwhelming

By reference to Table II it will be seen that of the 42 rabbits, each of which received 8 cc. of Serum 1, 22 animals, or 52 per cent, died. The rate fell well below this average in the case of the animals having the lowest bacteriemias while in the group of highest bacteriemias the average rate was much higher. The same type of result is also apparent in the column dealing with the deaths occurring within the

first 4 days. Similarly in the second series of 43 animals each of which received 4 cc. of Lot 2 the death rate was 65 per cent but the larger number of deaths occurred in the group of highest bacteriemias while the rate among those with the lower bacteriemias was much smaller. The same trend is shown among the animals which died early.

The data indicate that there is no absolute correlation between the severity of the infection as judged by the degree of the bacteriemia

TABLE II

Results of Scrum Therapy in Relation to the Bacteriemia at the Time of Treatment Each rabbit infected intradermally with Pneumococcus Type I. Designated amount of antipneumococcus serum administered intravenuosly 24 hours after infective inoculation. Degree of bacteriemia determined just before the administration of the specific serum.

Serum lot and amount given to		No. of pneumococci			Total deaths		Deaths within 4 days	
each rabbit	emia group	per cc. of blood	in group	No.	Per cent	No.	Per cent	
Lot 1, 8 cc.	A	0-200	5	1	20	0	0	
	В	200-2,000	9	4	44	2	22	
	С	2,000-20,000	15	6	40	5	33	
	D	Over 20,000	13	11	85	8	69	
	Entire g	roup	42	22	52	15	36	
Lot 2, 4 cc.	A	0–200	9	3	33	1	11	
·	В	200–2,000	8	4	50	3	38	
	С	2,000-20,000	11	7	63	6	55	
	D	Over 20,000	15	14	93	12	80	
	Entire g	roup	43	28	65	22	51	

and the result of subeffective serum therapy beyond the fact that those individuals with the more severe bacteriemias usually respond less well to the treatment. Perhaps certain of the discrepancies may be related to the technical difficulties in the exact determination of the number of pneumococci in the blood but even after due allowance is made for this possibility it remains a fact that all animals with infections of equal severity do not respond in the same way.

As these studies progressed it became increasingly apparent that some other factor or factors were involved in the results. In succeed----

ing series, determinations were made of the following variables: the white cell count at the time of infective inoculation and at the time of serum administration; body temperatures at all phases of the infection; the appearance of the local lesion as regards the area involved, the amount of edema, the intensity of the inflammatory color, and the occurrence of purpura; body weight; the coagulation time of the blood both at the time of infective inoculation and at the time of serum administration; the capacity of the rabbits' sera to agglutinate rough pneumococci; the heterophile antibody titer of the normal rabbit sera. In the case of only one of these was a suggestive correlation obtained, this being the white blood cell count at the time of serum administration.

Results of Serum Therapy in Relation to the White Cell Counts at the

Although it was seldom possible to correlate the white cell count at the time of serum administration with the outcome of the infection it is possible by a system of averages to show that animals which had a low white count had much less chance for recovery than did those rabbits which had a high white count. Such an analysis is presented in Table III.

The normal white cell count of rabbits varies within wide ranges, the average in our series being 11,920. As a rule this is considerably depressed during the earlier phases of the infectious process so that at 24 hours after infective inoculation the average is near 6,600 although here again there are many wide deviations from the mean. The animals have been divided into four groups on the basis of the white counts made at the time of serum administration so that the first group includes those animals with very low counts and the fourth group those with the very high counts. When the death rate in each of these groups is considered it is found that those animals with the very low counts died whereas those with high counts more frequently survived.

Thus with Serum Lot 1, the 3 animals with very low white counts died while 4 of the 5 with counts over 9,000 survived. The results are even more striking when the early deaths are considered. Similarly with Serum Lot 2, 4 animals with counts below 2,000 died whereas the death rate of those with high white cell counts was relatively low. Intermediate between these two extremes a definite difference is found when the comparative averages are considered but with the individual animal of any given group no significance

Since by the use of averages there seemed to be a definite significance attached to both the severity of the infection and to the white blood cell count at the time of serum administration a comparison was next made of the relation between the number of bacteria in the blood stream and the white cell count. Outside of the fact that animals with a very low white count generally also showed a very high bacteriemia no direct correlation exists between these two factors. How-

TABLE III

Results of Serum Therapy in Relation to the White Cell Count at the Time of Treatmen

Each rabbit infected intradermally with Pneumococcus Type I. Designated amount of antipneumococcus serum administered intravenously 24 hours after infective inoculation. White cell counts determined just before the administration of the specific serum.

Serum lot and amount given	White count	White cell counts No. of animals		Total deaths		Deaths within 4 days	
to each rabbit	group		in group	No.	Per cent	No.	Per cent
Lot 1, 8 cc.	а	Less than 2,000	3	3	100	3	100
	b	2,000-5,000	8	5	63	4	50
	c	5,000-9,000	6	1	17	1	17
	d	Over 9,000	5	1	20	0	0
	Entire	group	22	10	45	8	36
Lot 2, 4 cc.	a	Less than 2,000	4	4	100	4	100
	b	2,000-5,000	19	15	79	13	68
	c .	5,000-9,000	12	6	50	3	25
	d ·	Over 9,000	8	3	38	2	25
	Entire group		43	28	65	22	51

ever, if these two elements are considered in terms of the outcome of the infection a definite result is obtained. Such an analysis is shown in Table IV.

Table IV is compiled from the results obtained with subeffective amounts of three different lots of antipneumococcus sera of almost equivalent antibody content. Altogether 115 animals have been classified. This combination of results has been made in order to build up groups of significant numbers. It can be stated that the combined results are in every way similar to those of any one series.

The correlation between the white cell counts and height of bacteriemia with the result of the infection is shown in two parts, the first dealing with the total number of deaths, the second with only the early deaths. The significance of the two factors is quite apparent in the first part but is even more striking in the second.

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# Combined Summary of the Results of Serum Therapy in Relation to Bacteriemia and

This analysis includes the results of seven series of animals all of which were infected intradermally with Type I pneumococci. Each animal received a subeffective amount of specific antipneumococcus serum intravenously 24 hours after infective inoculation. Height of bacteriemia and white cell counts at the time of serum administration. (A) Analysis Based on Total Deaths

White cell counts	(A) Anal	ysis Based on T	otal Dean	at the tin
Less than 2,000 2,000-5,000 5,000-9,000 Over 9,000  Figures in bold fin ordinary type income the percentage of an	9 9 100 14 13 93 10 10 100	Bact 20,000-2,000 16 9 56	2,000-200 2 2 100	5 3 60 7 1 14 6 1 17

Figures in bold face type indicate total number of animals in group. Figures in ordinary type indicate number of deaths in group. Figures in italics indicate (B) Analysis Based on Deaths within 4 Days

	Analysis	Based on D	- ~	in italics indica
White cell counts		Death.	s within 4 Days	
	Over 20,000	Bact	deriemias Days	
Less than 2,000		20,000-2,000	1 125	
2,000-5,000 5,000-9,000	9 9 100	1,000	2,000-200	T
Over 9,000	10 0 93	16 8 50	2 1	200-0
	e " 00	10 5 50	13 6 46	_
Figures in bold fa ordinary type indica percentage of animal	Ce to	6 1 17	10 3 30	5 2 40
ordinary type indica percentage of animal	te numi	total	$\frac{1}{2}$ 0 0	7 0 0
e of anima	"""Der of do	an Humber of		600

Figures in bold face type indicate total number of animals in group. Figures in ordinary type indicate number of deaths within 4 days. Figures in italics indicate percentage of animals which died within 4 days.

All animals with very high bacteriemias and very low white counts died promptly whereas the opposite is true of those with low bacteriemias and high white counts.

In spite of the apparent significance of both of these factors no absolute prediction of the outcome in the individual case was possible.

These results appear to show that in the experimental dermal pneumococcus infection in rabbits both the number of organisms in the blood stream and the white blood cell count have a definite bearing on the curative action of specific antipneumococcus serum. Thus animals with low bacteriemias and high white cell counts responded well to specific serum therapy whereas those rabbits with low cell counts and high bacteriemias were apparently not benefited by the administration of the serum under the conditions of these experiments. Nevertheless it was impossible to predict with certainty the outcome in any individual instance. Certain factors of obvious importance were not controlled, first amongst them the breed of animals and thus indirectly the entire system of genetic host factors. Furthermore, rabbits of different weights were used.

## SUMMARY

In the experimental disease brought about by infecting rabbits intradermally with Type I Pneumococcus the use of relatively small or subeffective amounts of specific antipneumococcus serum leads to the survival of some individuals and the death of others. It has been shown that both the severity of the infection and the white blood cell count are factors in the determination of the outcome of the disease but that there remain other host factors which have to do with the utilization or functioning of the specific antisera.

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# STUDIES ON HOST FACTORS IN PNEUMOCOCCUS

# II. THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM

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The analysis of the experimental data presented in the preceding paper dealing with the curative action of specific antipneumococcus horse serum in Type I dermal pneumococcus infection in rabbits showed a dominance of two factors in determining the outcome of the infection; viz., the degree of bacteriemia, and the white blood cell count at the time of serum administration (1). There were, however, a large number of instances the results of which were not predictable on the basis of these two factors alone, and, although an exhaustive analysis was made of all available data, no other significant factors were found. As previously pointed out the complexity of this type of experiment admits of many technical errors. A further objection, in view of the obvious significance of the number of infecting organisms, was that the primary infection was focal and hence the estimation of the number of pneumococci in the blood stream could hardly be an adequate appraisal of the total number involved in the infection. It also became apparent as the work progressed that it would be absolutely essential to control genetic factors by the use of a single breed of rabbits.

These objections were overcome by the adoption of an experimental method consisting of a protection test in rabbits of a single breed, both antiserum and infecting organisms being injected at the same time intravenously. By this technic the variable genetic factors have been reduced, and the initial bacteriemia held constant.

Rabbits.—Throughout this series of experiments healthy male rabbits of the breed known as the New Zealand Red have been used. These animals are uniformly susceptible to Type I pneumococcus infection. Constant dietary conditions were maintained throughout these experiments.

Culture.—The strain of Pneumococcus Type I was of such virulence that 0.000,000,01 cc. of an 18 hour blood broth culture, given intradermally produces a fatal infection in normal rabbits. About 100 times this amount is required to establish infection if given by the intravenous route. Unless otherwise stated the culture was diluted 1–10 in broth and 0.5 cc. of this dilution used as the infective inoculum.

Scrum.—A single lot of Type I antipneumococcus horse serum was used throughout the experiments. Unless otherwise stated the amount injected was 0.5 cc. per rabbit.

Infective Inoculation.—Immediately after taking blood for the white cell count each rabbit received the serum intravenously in the right ear and the culture intravenously in the left ear, in the order named. The infective inoculation was invariably carried out at the same hour of the day.

Observations.—Since there is no visible external lesion following this infective procedure it was necessary to determine by the body temperature whether or not an infection had been established. Temperature determinations were made daily at the same hour on each rabbit. A rectal temperature of 104.0°F, or over was regarded as significant.

On the basis of the previous work it was expected that with this technically improved approach and a properly balanced combination of antiserum and microorganisms, all animals having a low white cell count would die while those with higher white counts would survive. This proved not to be the case, although it was found that 70 per cent of the individual animals having white counts below 10,000 died whereas 59 per cent of those with counts of 14,000 or over survived. On further analysis it was determined that the weight of the animal possessed considerable significance, since 65 per cent of animals under 2,000 gm. died whereas the death rate among the larger rabbits was only 39 per cent. The next step in the consideration of the results was to determine the relation between these two variables and the outcome.

For the purposes of this analysis all animals have been divided into three groups as follows:

Group A.—Rabbits which survived and which failed to show evidence of infection at any time.

Group B.—Those rabbits which survived but only after a febrile course indicative of the presence of an infective process.

Group C.—Those animals which developed an infection and died as a consequence thereof.

White blood cell count

15,260

11,100

16,600

18,550

12,000

13,640

27,250

10,080

18,900

15,040

12,300

16,100

12,100

19,400

10,150

6,450

12,350

15,240

17,840

17,050

23,800

7,680

13,850

11,250

7,400

20,100

6,850

7,500

11,520

9,200

11,600

13,550

11,900

11,760

13,400 16,720

Results of Protective Tests with Reference to Body Weights and White Blood

Each of the following rabbits received 0.5 cc. of Type I antipneumococcus serum

Body weight

ξm.

2,200

2,780

 $^{2,200}$ 

2,680

3,380

2,260

2,480

2,140

2,740

2,720

2,660

2,560

1,890

1,720

2,330

1,970

2,490

2,320

2,460

2,200

1,810

2,290

1,910

1,690

1,950

1,880

1,930

1,750

2,060

1,870

1,870

2,200

2,200

2,170

1,440

1,480

and 0.5 cc. of a 1-10 dilution of an 18 hour blood broth culture of Type I Pneumo-

Rabbit No.

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

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Completely

 $\mathbf{R}$ Animals which developed

an infection but even-

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C Animals which devel-

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died as a consequence

against infection

protected

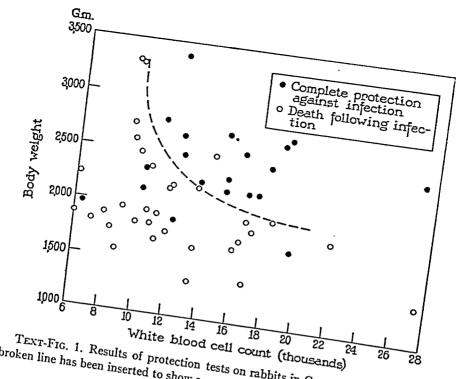
TABLE I-Concluded

Group	Rabbit No.	Body weight	White blood cell coun
C—Concluded Animals which developed an infection and died as a consequence thereof	6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34	1,980 2,470 3,280 1,950 1,860 1,370 3,300 2,230 1,830 1,960 1,890 2,580 1,650 2,510 1,920 1,680 1,570 1,770 1,760 1,950 2,730 1,820 1,870 1,690 1,760 1,830 1,840 2,220 2,340	18,250 9,750 9,350 16,600 17,000 27,400 9,050 6,140 10,750 8,950 7,850 9,300 13,450 14,320 11,050 15,960 9,700 11,750 8,350 10,500 9,240 7,050 6,000 11,050 16,040 9,700 21,860 4,300 10,950

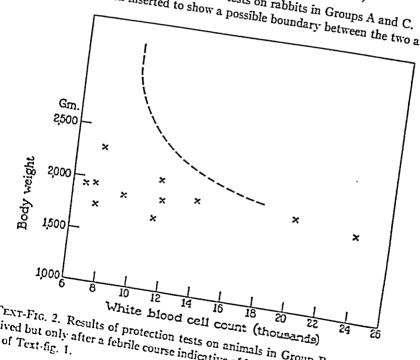
The results of the protection tests in the rabbits of these groups are listed in Table I together with the data which proved to be of the greatest significance as a result of the analysis.

By definition Groups A and C contrast the failure to establish any infection on the one hand against infection and death on the other. The results are plotted in Text-fig. 1 against the weight and the white blood cell count at the time of in-

A consideration of Text-fig. 1 shows that, in general, the members of Group A occupy one section of the figure while those of Group C are oppositely placed. A

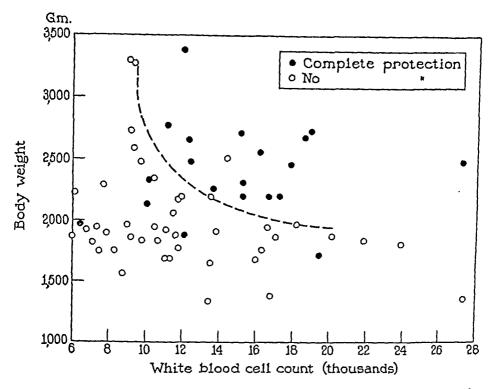


TEXT-FIG. 1. Results of protection tests on rabbits in Groups A and C. broken line has been inserted to show a possible boundary between the two areas.



TEXT-Fig. 2. Results of protection tests on animals in Group B. survived but only after a febrile course indicative of infection. The broken line is that of Text-fig. 1.

broken line has been inserted to show a possible boundary between these two zones. Six of the 65 points do not follow the general trend of distribution but the results are sufficiently definite to permit the conclusion that the heavier animals having white counts above the average seem to be completely protected against infection.



Text-Fig. 3. Text-figs. 1 and 2 are combined to show protection as against lack of protection. A broken line has been inserted to show a possible boundary between the two areas.

There remain for consideration the animals in Group B, all of which developed a definite infection but eventually recovered. The results have been plotted in Text-fig. 2 on the same scale as that used in Text-fig. 1 and the same broken line has been inserted in order to show the boundary that existed between Groups A and C. From a consideration of this figure it will be seen that all animals in Group B occupy positions in the area corresponding to those of Group C in Text-fig. 1. The natural division appears to be between animals which were protected against the development of infection as opposed

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to those which became infected. An analogy is to be found in a previously reported series of tests for active resistance in which it was shown that an animal which survived after a long febrile course could be considered as differing little from one which eventually died (2).

Hereafter in this paper the distinction will be made between protected as opposed to non-protected animals. On this basis Text-figs. 1 and 2 have been combined in Text-fig. 3, in which the animals fully Protected are shown by closed circles and those not protected by open

From these results it would appear that the weights and white cell counts serve as indices of two intrinsic variables which, under the present experimental conditions, appear to be significant when certain factors such as breed, amount of antiserum, and number of pneumococci are controlled. It appears that the heavier animals having the higher white cell counts are much more likely to be protected against infection. These results are somewhat surprising in view of the fact that the amount of serum injected was the same in all rabbits irrespective of the body weight. Under these circumstances the antibody concentration would be much greater in the smaller animals than in the larger. It seems reasonable to assume that the serum should be more effective as its concentration increases. to be the case. Consequently one must conclude that the larger animals possess a physiological capability which makes possible the This, however, proved not more effective utilization of the protective properties of the specific

## Significance of the White Blood Cell Count

Type of Cells Involved.—Differential counts have demonstrated that the only type of cell with which the results can be correlated is the polymorphonuclear leukocyte. Since the mechanism of the destruction of the pneumococcus undoubtedly involves phagocytosis this finding is not unexpected.

Variability of the Numbers of While Blood Cells in Rabbits.—It is quite generally recognized that the numbers of white blood cells vary widely in rabbits from time to time. A large variety of environmental and physiological conditions appear to influence the count of individual and physiological conditions appear to mindence the country individual animals to such an extent that within a single hour a considerable

shift may take place. In the present series the counts reported have been made immediately before the infective inoculation in each individual animal. Other counts made previous to this time and 1 hour, 4 hours, and 24 hours after the infective inoculation have shown little or no direct correlation. The results on a number of animals in the critical weight zone (2,000 gm. and over) are listed in Table II

TABLE II

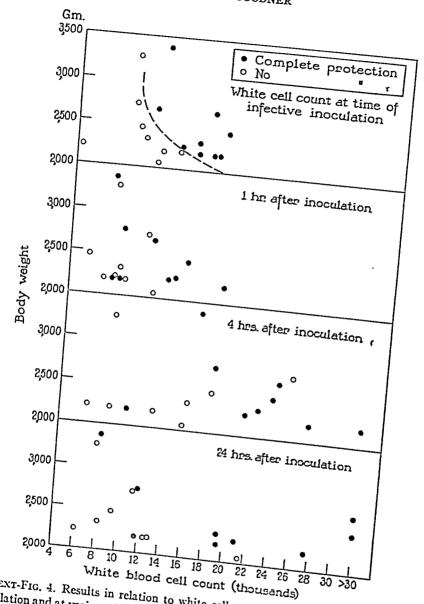
White Cell Counts at Various Times after Infective Inoculation

Each of the following rabbits received 0.5 cc. of Type I antipneumococcus serum and 0.5 cc. of a 1–10 dilution of an 18 hour blood broth culture of Type I Pneumococcus intravenously.

		_	Results in			
Rabbit	Body weight	Initial	After	terms of protection		
			1 hr.	4 hrs.	24 hrs.	
	gm.					[
а	2,060	11,520	11,900	15,400	21,600	-
ь	2,200	13,550	9,200	12,600	12,600	-
c	2,200	11,900	7,260	8,600	13,000	-
d	2,470	9,750	5,800	18,000	9,500	-
$\epsilon$	3,280	9,350	8,100	8,600	7,800	-
f	2,730	9,240	11,260	25,460	11,400	-
g	2,220	4,300	8,060	6,500	6,300	_
lı	2,340	10,280	8,700	15,800	8,300	_
i	2,200	17,050	18,400	30,700	27,600	+
$\boldsymbol{j}$	2,460	17,840	14,900	23,704	74,800	+
$\boldsymbol{k}$	2,320	16,240	13,200	22,400	19,460	+
ı	2,260	13,640	13,900	21,200	21,100	+
111	3,380	12,000	7,940	16,600	8,100	+
n	2,680	18,550	11,800	24,200	31,000	+
0	2,200	16,600	8,700	27,200	11,800	+
Þ	2,780	11,100	8,900	18,200	11,800	+
$\overline{q}$	2,200	15,260	8,000	10,200	19,500	+

<sup>-</sup> = no protection. + = complete protection.

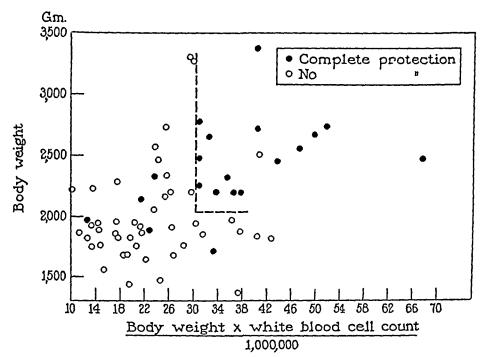
and plotted in Text-fig. 4. From these results it will be seen that after the time of infective inoculation there is little apparent organization of the points into definite characteristic areas. The possible significance of these findings will be discussed later, but it may be stated that the results indicate that the only white cell count of significance is that made just at the time of infective inoculation.



TEXT-FIG. 4. Results in relation to white cell counts at the time of infective inoculation and at various times thereafter.

The Total Number of White Blood Cells.—From the data shown in Text-fig. 3 it appears that an animal of 3,000 gm. is protected if the

white count is over 10,000 but that an animal of 2,000 gm. requires a much higher count for protection. Obviously a large rabbit actually has a much greater total number of circulating white blood cells than a smaller animal having the same count. This suggests that the result may actually be determined by the total number of white cells. This, of course, cannot be absolutely determined but if one multiplies the cell count by the body weight in each instance, one arrives at a figure representing a relative value for the total number of white cells



Text-Fig. 5. Results plotted against weight on the basis of relative total white blood cells.

in the blood stream. This calculation has been carried out for the animals shown in Text-fig. 3 and the results plotted against the weight are shown in Text-fig. 5. From a consideration of the data it would appear that the results are divided sharply into two divisions, and that the curve of Text-fig. 3 has been replaced by straight lines, one representing a minimum absolute number of white blood cells and the other a minimum weight. The significance of these minimums will be discussed later.

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of B. coli were given to several animals. A suitable amount of the suspension was judged to be one which would produce a considerable

## Induced Leukocytosis in Reference to the Protective Action of Antipneumococcus

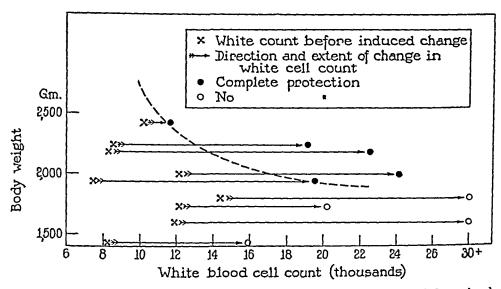
Each of the following rabbits received an intravenous injection of a heat-killed suspension of B. coli representing 0.2 cc. of a broth culture. 18 hours thereafter each animal received 0.5 cc. of Type I antipneumococcus serum and 0.5 cc. of an 18 hour blood broth culture of Type I pneumococcus intravenously.

	- 510	n culture of	T- an	tipneumo	Con-	hours then
Rabbit	Body weight	Prelin	Type I	рпеитосо	coccus serum	hours thereafter and 0.5 cc. o.
A B C D E F G	fm. 2,540 2,420 2,240 2,180 2,000 1,940	7,150 10,300 8,700 8,300 12,150 7,340	coli suspension injected intravenously	18 hrs.	White cell contributed in the cell cell cell cell cell cell cell ce	int
H I J		14,460 12,100	suspensi	Interval of	24,100 19,450	++++
- = no pro	1	11,900	~· 1		35,860 20,400 50,400	
leukocytosis		= complete p	protection	1.	15,850	

leukocytosis yet not prove fatal. Preliminary counts were taken on a number of animals and the bacterial suspension then administered intravenously. 18 hours thereafter the regular infective procedure was carried out. The results of these experiments are listed in Table III and are plotted in Text-fig. 6. In this figure the counts before the change induced by the injection of the heat-killed suspension of B. coli are indicated by crosses while the counts at the time of the infective inoculation are shown by the circles. These points are joined by

lines in order to show the direction of the shift in count. The curve is that shown in Text-fig. 3.

It is apparent from these results that the non-specific stimulation produced in all cases a leukocytosis and that if the shift was sufficient to place the individual within the so called protective zone the result was complete protection. This treatment did not, however, induce any other apparent change since animals which lacked the required weight were not protected. These data support the previous deduction that the outcome is directly related to the cellular status at the time of infective inoculation.



Text-Fig. 6. Induced leukocytosis and the effect on the capacity of the animal to be protected against infection. The broken line is that of Text-fig. 3.

It is impossible at the present time to construct a hypothesis which will explain these findings. A rough calculation shows that the proportional number of white cells is about 200 for each pneumococcus injected. This ratio is surprising for in *in vitro* experiments many polymorphonuclear cells often take up several pneumococci each. It has been suggested that the ratio may be determined by the chances of collision between the cells and the pneumococci in the circulating blood but due to the probable participation of fixed or stagnant phagocytic cells this hypothesis is difficult to substantiate. It has also been

Significance of the Weight Factor It is a general impression that weight is an expression of age. fortunately, the exact age of none of these animals is accurately known and consequently this hypothesis, although probably correct, cannot

be definitely supported. If this were true one might more properly

The results of experiments now in progress indicate that the weight factor is associated with some passively transferable component of the serum. Although our information is as yet too meager to warrant definite conclusions this factor appears to be heat-labile but not iden-

Interrelationships of Intrinsic and Extrinsic Factors It would seem important to learn in what manner the various dominant factors might be mutually related, but even by maintaining the genetic elements constant this is a difficult task. The situation is one in which four variable factors may be related each to the other. With the amount of antiserum and the number of infecting organisms constant it has been shown that an increase of white cells does not compensate for a lack of weight and that an excess of weight does not compensate for a lack of white blood cells. In so far as our experience extends, there is no reason to believe that the weight and white cell

The further approach to this question is through the experimental

use the term physiological maturation.

tical with the so called hemolytic complement.

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count are mutually compensatory or related in any way except through variation of one of the extrinsic factors, keeping the other and one of the intrinsic elements as nearly constant as possible.

is not difficult but the results are confusing since a variation in one

of the extrinsic factors immediately upsets the delicate balance that had been created. Nevertheless the results that have been obtained

are presented and the possible significance considered briefly.

Variations in the Amount of Specific Antipneumococcus Serum in Relation to the Body Weight Factor.—If the amount of specific antipneumococcus serum is varied and the white count maintained a con-

the mediation of the two extrinsic factors.

This approach

stant by the selection of animals showing counts within a narrow range, it is possible to obtain a result showing the interrelation of serum and weight. Thus in a range of white cell counts of 11,000 to 12,500, if 0.5 cc. of the serum was employed, the minimal weight at which protection was obtained was about 2,300 gm., with 0.4 cc. of serum it was approximately 2,900 gm., while with 0.6 cc. of serum the minimal

TABLE IV

The Relation of Number of Pucumococci in the Infective Inoculum to the Number of White Blood Cells in Reference to the Protective Action of Antipneumococcus Scrum

		Infecti	ve inocula					
	0 05 cc.			0,005 cc				
Weight	White count	Result	Weight	Weight White count				
gm.			gm.					
			2,060	8,760	_			
	}		2,160	9,960	_			
2,140	10,080	+	2,080	10,400	_			
2,060	11,520		2,060	11,000	+ +			
2,170	11,760	_	2,050	11,550	+			
2,200	11,900			}				
			2,140	12,300	+			
2,200	13,550	_		1				
	1		2,100	14,080	+			
	<b>{</b>		2,010	14,520	+			
2,200	15,260	+		1 1				
2,200	16,600	+	1	1				
2,200	17,050	+	1	1				
			2,140	20,600	+			

<sup>-</sup> = no protection. + = complete protection.

weight was 1,700 gm. These experiments, too extensive to be reported here in detail, have conclusively shown that increasing amounts of serum can compensate for lack of weight and *vice versa*.

Variations in the Amount of Specific Antipneumococcus Scrum in Relation to the White Cell Count Factor.—No evidence has as yet been obtained which would indicate that increasing amounts of serum compensate in any way for a comparative lack of white blood cells.

KENNETH GOODNER Thus even when 2.5 cc. of the serum, or 5 times the standard amount ---.... was used, the white cell minimum remained the same with the standard 115 number of infecting microorganisms. 7.

Variations in the Number of Pneumococci in Relation to the White Blood Cell Factor.—In another series of experiments the amount of serum was maintained constant but the amount of culture was decreased to 0.005 cc., or one-tenth the amount of culture previously injected. Then by a comparison of results in a selected weight range, the relation of this change to the white counts can be determined. The results of such an experiment are shown in Table IV together with data on animals of comparable weight in the previous series. From the table it would appear that a decrease in the number of pneumococci permits the protection of those animals with lower white counts. Hence the number of white blood cells necessary is definitely related to the number of pneumococci, more cells being required with increasing numbers of infecting organisms, although not in the same ratio.

Variations in the Number of Pneumococci in Relation to the Weight Factor.—From the data now available it seems certain that, as in the previous experiment, when relatively few pneumococci are injected, animals with lower white cell counts and also those of lower weights are protected. Under such circumstances all rabbits with white cell counts above the average, no matter how small they themselves were as individuals, appeared to be protected. This is, we believe, not due to any direct correlation between the number of bacteria and the weight of the rabbit but to the fact that the intimate balance between number of pneumococci and the amount of serum has been disturbed. It has already been shown that changes of as little as 0.1 cc. in the amount of serum make a considerable difference with regard to the minimal weight. It is not surprising therefore that with the decreased amount of culture, corresponding proportionally to a tenfold increase in the amount of serum, the smaller animals were protected. The result is believed to be a reflection of the altered serum-culture ratio rather than a direct effect.

In so far as can now be determined the relationship of these variable factors, the breed being constant, may be illustrated by the following diagram:

The intrinsic factors appear to be related in this manner to the extrinsic factors and through these to each other.

#### DISCUSSION

The purpose of these experiments has been to arrange critical combinations of controllable variables in order to learn, if possible, whether the results might be in any way related to one or more determinable host factors. As the work progressed it has been possible to make certain technical improvements and thereby bring out certain more dominant factors. In the first paper (1) experiments were reported which sufficed to point to the present more successful method of approach. It should also be mentioned that the normal rabbit is completely lacking in any form of specific antipneumococcus antibodies so that the quantity of antiserum administered represents the complete amount of specific immune substances present. Furthermore the rabbit is so susceptible to Type I pneumococcus infection that the results are sharply defined.

The experiments appear to have demonstrated the existence of two host factors which have a direct bearing on the capacity of the animal to utilize specific antipneumococcus antibodies. These two intrinsic factors find expression in terms of body weight and white blood cell count; but no evidence has been obtained to show that either the weight or the number of cells is other than a reflection of a more complex physiological system. They serve, however, as indices and, as such, they express the quantitative aspects of non-specific host resistance. Under the circumstances of the present experiments it has been possible to demonstrate that heavier animals with high white cell counts are in a physiological condition favorable to the utilization of passively conferred specific antibodies. It would appear that those animals which are physiologically mature and possess a condition of

KENNETH GOODNER high cellular reactivity as evidenced by the number of circulating white blood cells have a considerable advantage in the utilization of antibacterial antibodies over animals less mature and with a lower cellular activity.

The power of specific antipneumococcus serum to protect rabbits against infection with Type I Pneumococcus has been studied with reference to the capacity of the animal to utilize the specific antibodies. Under conditions ensuring relatively controlled genetic factors it was found that heavier animals and those with high white blood cell counts are much better able to utilize the passively conferred immune principles. The interrelationships of the extrinsic and intrinsic factors responsible for immunity have been discussed.

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### PATHOLOGIC HISTOLOGY OF THE SHWARTZMAN PHENOMENON WITH INTERPRETATIVE COMMENTS\*

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#### PLATES 1 AND 2

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The exact nature of the processes involved in the Shwartzman phenomenon is as yet not settled. This study of the histology of the reaction is presented in an attempt to arrive at a better understanding of the phenomenon. The studies of Karsner, Ecker and Jackson and of Moritz and Morley demonstrated that the Shwartzman reaction can be produced in stomach and knee joints of rabbits respectively. The details of the technique are given in their publications. The material obtained has been studied in the gross and microscopically. It was shown that the reaction in the stomach closely parallels the skin reactions in the same animals but the same was not true of the knee joints.

#### EXPERIMENTAL

Four control rabbits were employed in the stomach series. These were killed by fracture of the neck at 7 hours, 24 hours and two at 7 days after the preparatory injection of concentrated toxic filtrate of B. coli culture into the submucosa. In the gross, the only evidence of change at the site of inoculation in the 7 and 24 hour periods was slight swelling of the mucosa. In the two animals killed at 7 days the mucosa over areas of 8 by 10 and 8 by 12 mm. respectively was swollen and red and on section showed a gelatinous character of mucosa and submucosa. Sections were fixed in both 10 per cent neutral formalin and in Zenker's fluid, embedded in paraffin, cut at 6 micra and stained with hematoxylin and eosin. Microscopically the 7 hour stomach shows hemorrhage in the peritoneal and muscular coats, almost certainly traumatic. The submucosa is the seat of edema, fibrin formation and the infiltration of a few polymorphonuclear leucocytes and a

<sup>\*</sup> Aided by a grant from the Josiah Macy, Jr., Foundation.

few mononuclear cells principally of lymphocytic type. Capillaries are large but hyperemia is not a prominent feature. In the 24 hour stomach, traumatic hemorrhage is not observed. Edema of the submucosa with fibrin formation is well marked. In this area are many polymorphonuclear leucocytes with a few large and small mononuclear cells. The leucocytic infiltration involves the neighboring mucosa, without injury to the muscularis mucosae and also extends along the septa of the inner and outer muscular coats of the organ. The state of the blood vessels is about the same as in the 7 hour animal. Both the 7 day stomachs showed essentially the same features microscopically. The edema is still present, but the polymorphonuclear leucocytes are much reduced in number. Large and small mononuclear cells are in larger numbers than before. There is a large amount of granulation tissue with fibroblasts, often multipolar, and newly formed capillaries. In the septa of the muscular coat of the organ are lymphocytes, large mononuclear cells and fibroblasts.

Thus it can be said that the preparatory injection of toxic filtrate into the submucosa initiates an inflammatory reaction within a few hours, characterized by edema, fibrin formation and cellular exudation. Eosinophiles with their large cytoplasmic granules are not found and the vascular change is only occasionally productive of hemorrhage.

Seven control animals were used in the study of the Shwartzman reaction in the knee joints of rabbits. In these the preparatory factor was injected into the knee joint but as in the stomach controls, the subsequent intravenous injection of the reacting factor was not made.

These animals were killed from 25 to 222 hours after injection, which was followed in all instances by an acute synovitis. The inflammation is characterized by hyperemia, slight edema and exudation of small and large mononuclear cells, the former predominating. This reaction is most pronounced in the immediate vicinity of the injection. Polymorphonuclear leucocytes and fibrin vary considerably in amounts but as a rule are inconspicuous elements of the exudate. Hemorrhage is usually confined to the region of the puncture wound. The synovial lining cells are swollen and in some instances hyperplastic. The impression is gained that the joint fluid is increased or is at least under greater pressure than normal. Smears of joint fluid reveal only an occasional mononuclear cell.

There is considerable variation in the extent and severity of the synovial reaction to the preparatory factor; a variation that is not entirely related to the time that has elapsed after injection. The inflammation is present in all four animals killed between 25 and 30 hours after injection but in two of these the reaction is mild and in the other two quite severe. In the three animals sacrificed at longer

periods after the injection, the inflammatory response varies in a manner that is not consistent with the time interval. Edema and hyperemia are less severe after 72 hours than under 30 hours.

In the stomach series the experimental animals were killed at periods of  $3\frac{1}{2}$ , 5 (two animals), 12, 24, 25 (two), 72 (two) hours, and 7 days (two) after the intravenous injections. The reactions in the stomach were much the same as those of the skin of the same animals except that the peak of the reaction in the skin was reached in 4 or 5 hours and in the stomach at about 24 hours.

As early as  $3\frac{1}{2}$  hours after the intravenous injection the edema of the submucosa is much more marked than in the controls, the amount of fibrin appears to be greater and the cellular infiltration is severe. The cells are mainly polymorphonuclear leucocytes but there is a considerable number of small and large mononuclear cells, the latter phagocytic for nuclear fragments and erythrocytes. The cellular exudate extends into the muscularis mucosae and the tunica muscularis. Hyperemia is moderate and small hemorrhages are observed. The peritoneum is normal and the mucosa shows no necrosis.

At 5 hours, the changes are much the same, except that the hyperemia is more marked and there is occasional venous thrombosis, largely fibrinous.

At 12 hours, the fibrin shows numerous asters and the cells of the exudate show necrosis, as evidenced by marked karyorrhexis.

At 24 and 25 hours, the changes are found in great intensity, with considerable hyperemia and hemorrhage, venous thrombosis, exudate, now more richly cellular, marked karyorrhexis and noteworthy phagocytosis. In all three of these animals the number of large mononuclears is greater than before, but polymorphonuclears still predominate. A few fibroblasts appear in the submucosa and in one case in the septa of the tunica muscularis.

At 72 hours, the amount of edema is reduced, the fibrin condensed in bands and the number of polymorphonuclears both relatively and absolutely decreased. Phagocytosis and karyorrhexis are still present. The predominant cells are large and small mononuclears. The fibroblasts have increased considerably in number and there are so many thin walled capillaries as compared with the controls that they must represent capillary multiplication. Simply put, granulation is

At the end of 7 days, edema has not entirely disappeared and there are still a few polymorphonuclear, and large and small mononuclear cells. The fibroblasts are present in great numbers and there is much collagen, but the capillaries are less numerous than at the 72 hour period. There is then definite progress in the direction of cicatrization.

In addition to the sections from the actual areas of injection, sections were taken from other parts of the stomach wall, both in the control and experimental animals. These show reactions of the same general character, but far less marked in degree. In none of these was thrombosis or hemorrhage observed. It appears that the severe local reactions are accompanied by less marked but similar (not identical) changes throughout the stomach wall.

In no instance was anything even remotely suggestive of mucosal necrosis or ulceration observed.

As regards the knee joints, six of the eleven animals in which both preparatory and reacting factors were injected reveal a local inflammatory reaction which is definitely more extensive and which differs in character from that seen in the controls. In the other five, the inflammation is not in excess of that present in the controls. taneous Shwartzman reaction was attempted in skin and synovia of six rabbits, and although the skin reaction varies in severity and is consistently more severe than that present in the synovia there is no correspondence between the severity of skin and synovial reactions in the same animal. The Shwartzman reaction in the skin, stomach and synovia is qualitatively similar in that in all three situations a positive reaction is characterized by endovascular necrosis, thrombosis, hemorrhage and exudation of polymorphonuclear leucocytes and fibrin. The macroscopic changes in the joints are not significantly different from those seen in the controls and are characterized by swelling and hyperemia of the synovia. In several of the more severe reactions the joint fluid is blood-tinged and under increased pressure, but smears of this fluid do not reveal any appreciable increase in cells.

The Shwartzman reaction in the joints appears to be fully developed so far as thrombosis and hemorrhage are concerned as soon as 1 hour after injection of the reacting factor, this being a considerably prompter reaction than is elicited in the skin. Exudation with necrosis of both fixed tissue and exudate is increasingly pronounced in 5 to 10 hours after injection of the reacting factor. Fibroblasts and granulation tissue are found at 25 and 26 hours after injection of reacting factor, but organization is not extensive even after 196 hours.

Skin sections have been described by Shwartzman, but without note as to the reaction which follows injection of the preparatory factor. Skin sections in our animals show essentially the same type of inflammatory reaction as in stomach and joints, except that in the skin vascular thrombosis is more frequent and hemorrhage more extensive.

Summary.—The preliminary injection of filtrates used in the Shwartzman phenomenon leads to exudative inflammation, which although of variable degree, is usually slight or mild. Following the subsequent intravenous injection of the filtrates, the inflammation becomes much more severe and is accompanied by vascular thrombosis, vascular necrosis and hemorrhage. The exudate is in large part inflammatory edema and at the height of the reaction the cells are predominantly polymorphonuclear leucocytes. Necrosis of exudate occurs and there is notable phagocytic activity by large mononuclear cells and by polymorphonuclear leucocytes. Eosinophiles are not found at any time. The process heals by granulation, organization and cicatrization.

#### DISCUSSION

There is little doubt that the Shwartzman phenomenon belongs somewhere in the category of immune processes. Shwartzman separated it from allergic phenomena by the local reactivity, short incubation, short duration of reactivity, initiation by a single skin injection, severity of reaction and necessity for intravenous injection of the reacting factor. Hanger thought that skin reactions to filtrates are due to previous bacterial sensitization. Gratia and Linz were definitely of the opinion that the Shwartzman and Arthus phenomena are related. This they based on observations that (1) in certain cases where the Shwartzman is particularly intense it is accompanied by general changes like those of anaphylactic shock, such as capillary stasis, fall of blood pressure, thrombopenia and delayed coagulation of blood corrected by a current of CO<sub>2</sub>; (2) the Shwartzman phenomenon when especially intense may desensitize against anaphylactic shock; (3) the rat and mouse are refractory to both Shwartzman and anaphylaxis;

(4) in spite of differences in bacteria, filtrates and primary effect, the same hemorrhagic reaction follows the intravenous injection and this can be exactly duplicated in a modified Arthus phenomenon. Their views are not necessarily controverted by the fact that they do not distinguish true anaphylactic shock and anaphylactoid phenomena. The following discussion concerns the question as to whether or not the morphological study of the process can throw light on the subject.

Reports of the effects of the primary injection show important differ-Although Shwartzman stated that the local injection excites little or no reaction, a view shared by Apitz and by Gratia and Linz, microscopic examinations were not published.1 Cohen found it impossible to elicit the Shwartzman phenomenon in the lungs without previous bacterial injury of those organs. Our results indicate plainly that inflammation is produced by the primary local injection, visible in the gross and microscopically. The severity of these reactions may well be due to the fact that the filtrates employed were concentrated. The concentrates from synthetic medium cultures were made by the method of Ecker and Rimington. Ecker and Welch reported severe local reactions to these concentrates and were confident that their product contained the essential factors necessary to the Shwartzman phenomenon. That reactions may be produced by filtrates which have not been concentrated is beyond doubt. Hanger observed strong skin reactions in previously untreated rabbits with 72 hour broth culture filtrates of Bacillus lepisepticus. Teissier and collaborators found similar reactions in guinea pigs to pertussis preparations. Unconcentrated filtrates are primarily toxic when administered intravenously, as shown by Gratia and Linz. Concentrates were found to be toxic by Menten and King and Menten and Kipp. P. Bordet employed filtrate concentrates (acetic acid precipitation and solution of the precipitate) and arrived at the conclusion that primary toxicity intervenes in the determination of the Shwartzman phenomenon.

<sup>&</sup>lt;sup>1</sup> Kielanowski and Selzer (Kielanowski, T., and Selzer, A., Compt. rend. Soc. biol., 1934, 115, 648) have reported, as a result of the histological study of the skin of ten rabbits, that the preparatory factor produces inflammation. This does not differ in animals susceptible or refractory to the final reaction. They attribute the hemorrhage to humoral changes which provoke increased intracapillary pressure and rupture. These statements require confirmation.

This is not necessarily contradicted by the observation of Julianelle and Reimann that the purpura-producing substance of pneumococcus extracts is present in the supernatant fluid after acetic acid precipitation. Burnet thought that a variety of anaphylaxis determines the toxicity of intravenous injections, but the evidence is not convincing and his autopsy findings are like those of Bordet in rabbits not previously treated; namely, marked hyperemia of the lungs with small hemorrhages. In the Sanarelli phenomenon, hyperemia and hemorrhage are found but principally in the intestines. There can be no doubt that the preparatory factor of Shwartzman is irritant and poisonous but the effects vary with the degree of concentration, and possibly also with the degree of reactivity of the animal.

In respect of inflammatory response to the first injection the Shwartzman does not differ significantly from the Arthus, for although reaction to the first injection was not noted by Arthus and Breton, it has been amply demonstrated by Gerlach, Opie and others.

The question then arises as to whether or not this primary skin reaction in the Shwartzman phenomenon can be identified anatomically as allergic. The views of Gratia and Linz in this connection have been abstracted above. Previously, Hanger indicated that the local reactions to products of the Gram-negative organisms is due to sensitization of the animals to these organisms commonly present in the upper respiratory tract, whereas P. Bordet referred the sensitization to similar organisms in the intestinal canal. Hanger observed occasional reactions to filtrates of streptococcus cultures and thought this represented sensitization to those organisms. Shwartzman reported irregularity in response to streptococci but Burnet observed no response. The relatively refractory state of guinea pigs was explained by Hanger as due to low incidence of Gram-negative organisms in the respiratory tract of these animals.

If the reaction is to be classed as allergic on anatomic grounds, characteristic criteria of allergic inflammations must be established. Although edema, collagenous swelling, fibrinoid change and eosinophilia have been thought by some to be specific, the case is not proven, for they occur in other types of lesion. Nordmann found a total lack of agreement as to changes in connective tissue cells. There also appear to be differences in various animals. Eosinophiles were found by

Rössle in hypersensitive reactions in the guinea pig, but not by Dienes and Mallory. Davidoff, Seegal and Seegal reported a few cosinophiles in anaphylactic inflammation of rabbit brain, but Seegal, Seegal and Jost did not observe them in similar lesions of the pericardium. Kline, Cohen and Rudolph and others have observed large numbers of eosinophiles in allergic skin reactions in man. In the rabbit skin, however, Gerlach did not find them and Opie reported them only following simultaneous injection of antigen and antibody. We found none in the Shwartzman phenomenon in the rabbit. As noted above, the primary inflammatory reaction of the Shwartzman shows edema, vascular lesions, leucocyte and mononuclear cell infiltration, but there is nothing that would prove whether this is allergic or not. Fibrinoid is reported in allergic reactions by Gerlach and others of the Rössle school. When present it is significant but not necessarily pathognomonic for it may be found in other conditions. Therefore, the absence of fibrinoid in our experiments does not exclude the possibility that the reaction is allergic.

Evidence that the inflammatory reaction produced by local injection of the preparatory factor in the Shwartzman phenomenon is allergic in nature is based upon physiological and immunological experiments. The morphological studies here reported are in accord with the assumption but not conclusive evidence.

The local reaction which follows intravenous injection of the reacting or provocative factor differs from that of the local injection or preparatory factor quantitatively rather than qualitatively. The exudative inflammation is accompanied by hemorrhage and necrosis. The hemorrhage is greater than that observed in the classical Arthus phenomenon and the necrosis less. The collagenous swelling occurs in both but is more prominent in the Arthus phenomenon. The essential features are the same in both phenomena save for these quantitative differences. Eosinophiles were not observed in the reaction in our animals. Blood vascular degeneration, inflammation and thrombosis were more prominent following the intravenous injection than after the local injection. As regards the local injection, however, these vascular changes were more pronounced in the periarticular structures than in stomach or skin. That vascular injury occurs in the Arthus phenomenon is plainly shown by Opie, and in a modified

HOWARD T. KARSNER AND ALAN R. MORITZ technique for the production of the Arthus, Gratia and Linz report a hemorrhagic reaction which they claim is indistinguishable from the E <u>ښ</u>:

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Since the local injection of preparatory factor excites inflammation it is probable that the intravenous injection leads to an increased concentration of injurious substance at the local site. Auer's experiments showed that local inflammation may bring about a concentration at that site of antigen-antibody complexes. Opie found that in the immune animal, protein injected locally is fixed at the site as contrasted with the rapid diffusion in normal animals. Furthermore the local fixation rapidly leads to destruction of the protein. (1930) showed that whereas protein is diffused out of inflamed areas with difficulty, it enters readily from the blood stream and is concentrated at the site of inflammation. He (1931) quoted Landis as demonstrating that injured capillaries are rendered extremely permeable and that the capillary pressure is increased in inflamed areas. This explains the ready entry of proteins from the circulation into the area of inflammation.

The exact chemical nature of the factors in the Shwartzman phenomenon is not known. The acetic acid precipitate employed by Bordet is made up of proteins and perhaps glycoproteins. terial we used was made by the method of Ecker and Rimington. They found in similar preparations that the nitrogen content was from 0.3 to 0.4 per cent and found positive color reactions for carbohydrates, suggesting that the material is largely polysaccharides, certainly with extremely little protein. It seems probable therefore that the factors in the Shwartzman phenomenon are not necessarily rich in protein. Thus the reaction would not be of exactly the same nature as the Arthus. As pointed out by Ecker and Rimington it is impossible to say whether the carbohydrate is a carrier of the toxic substance, a colloid impurity or part of the molecule of the toxic compound. Using a 1 per cent peptone broth instead of synthetic medium, Menten and King produced a concentrate which, like that of Ecker and Rimington, is principally carbohydrate in nature. Both Menten and King and also Burnet regard the effective substance as an endotoxin. Certain experiments point to an especial vulnerability of the blood vessels to the factors involved in the Shwartzman phenomenon.

Thus, as shown by Shwartzman and Michailovsky in mouse sarcoma, and by Gratia and Linz in guinea pig liposarcoma, the vessels of certain transplantable tumors are injured by intravenous injections of the filtrates. This has been observed in animals not susceptible to the Shwartzman and, as reported by Duran-Reynals, applies especially to rapidly growing and highly malignant transplantable tumors. Shwartzman regarded the vascular vulnerability as transient.

The intimate relation of the phenomenon to vascular lesions is further indicated by the fact shown by Peck and Sobotka and by Peck, that certain snake venoms can produce a refractory state to the Shwartzman phenomenon. It may then be said that although the chemical nature of the essential factors is not known they probably are related in some way to the carbohydrates, but whatever the exact composition may prove to be the action is largely in the direction of vascular injury.

It is no longer necessary to assume that immunity and sensitization are related to proteins only. Consequently the factors in the Shwartzman phenomenon cannot be excluded as reacting substances in a sensitive animal. It is therefore conceivable that those animals which exhibit the Shwartzman phenomenon have been sensitized to products of Gram-negative and rarely other bacteria, that the reaction to the preparatory factor is in part due to this sensitization and that the augmented severity of the reaction following intravenous inoculation is due to concentration of the factors in the inflamed site of the preparatory local injection. There must, however, be other conditions involved. Although Gratia and Linz claimed that ricin can act as a preparatory factor, they obtained negative results with distilled water and with 20 per cent NaCl solution. Shwartzman reported negative results with a variety of control substances including turpentine. Thus, the preparatory injection must not only produce inflammation but also effect a particular state of vulnerability of the blood vessels. Since no differences are demonstrated between preparatory factor and provocative factor, except that the latter may be neutralized by immune serum, the two factors are probably one and the same, and the essential effects of both on blood vessels are probably identical.

#### CONCLUSIONS

The histological study of the reaction to injection of the preparatory factor of the Shwartzman phenomenon shows it to be an exudative inflammation, but furnishes no conclusive evidence as to whether or not the inflammation is conditioned by previous sensitization to substances contained in the bacterial filtrate.

The histological study of the local reaction which follows the intravenous injection of reacting or provocative factor shows exudative inflammation which differs from that due to the preparatory factor principally in the increased general severity and marked damage to blood vessels. The increased severity of reaction which follows the intravenous injection is probably due to concentration of the injurious agent at the site of inflammation determined by the preparatory local injection.

The exudative phenomena caused by the preparatory local injection are edema and infiltration of polymorphonuclear leucocytes and large mononuclear cells. Vascular injury is morphologically demonstrable in only a few of the sections, notably those from periarticular structures. The intravenous injection determines an increase in cellular infiltration, necrosis of exudate, phagocytosis of cell debris, destruction of vascular walls and hemorrhage. Healing is due to granulation, organization and cicatrization.

Grateful acknowledgment is made to Professor E. E. Ecker for aid in this study.

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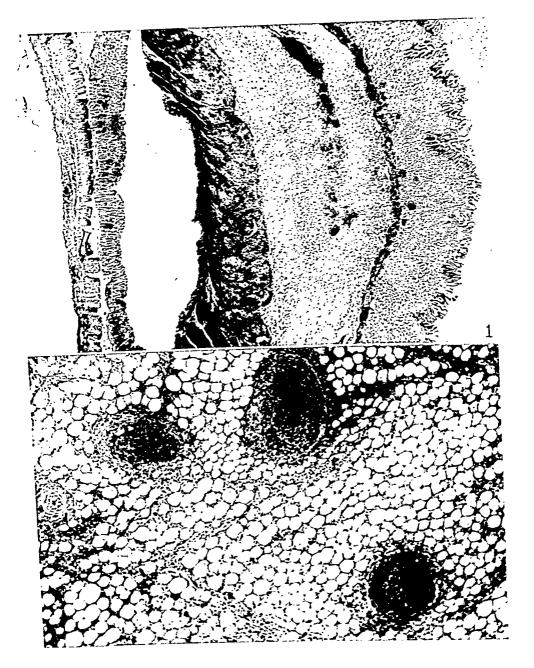
#### EXPLANATION OF PLATES

#### PLATE 1

- Fig. 1. Stomach wall 7 days after intravenous injection of reacting or provocative factor. Severe edema of submucosa and in higher magnification granulation and organization are present. At the left a remote normal part of the same stomach. Hematoxylin and eosin.  $\times$  20.
- Fig. 2. Hyperemia, exudation and venous thrombosis in anterior fat pad of knee joint, 4 hours after intravenous injection of provocative factor. Small artery near margin is unaffected.  $\times$  94.

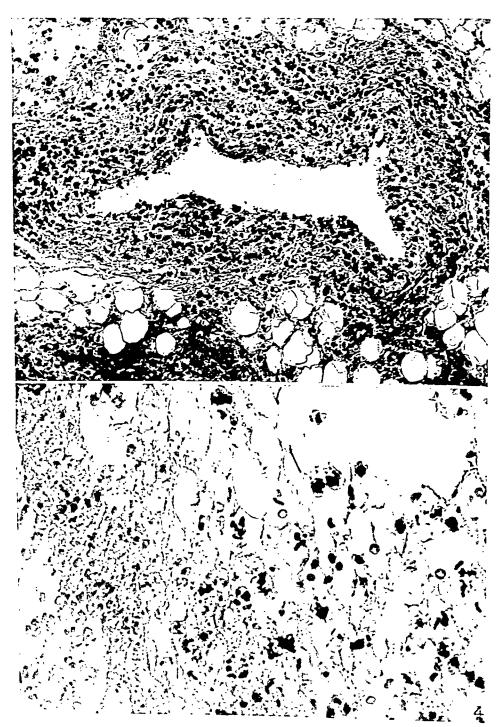
#### PLATE 2

- Fig. 3. Exudation in wall of small vein in anterior fat pad of knee joint, 22 hours after local injection of preparatory factor. Control observation. Reaction more severe than usual. Hematoxylin and eosin. × 252.
- Fig. 4. Necrosis of exudate and phagocytosis of cell fragments in submucosa of stomach, 25 hours after intravenous injection of provocative factor. Hematoxylin and eosin. × 660.



(Karsner and Moritz: Histobary of Shwartzman pher menon)





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# THE INFECTION OF FERRETS WITH SWINE INFLUENZA

(From the Department of Animal and Plant Pathology of The Rockefeller Institute PLATE 3

(Received for publication, March 27, 1934)

Smith, Andrewes, and Laidlaw (1) have obtained, from cases of influenza in man, a virus which is pathogenic for ferrets. They further observed that the virus of swine influenza was also infectious for ferrets and produced in these animals a disease similar to that caused by the virus of human origin. The present paper confirms and extends the observations of the above authors regarding the infectivity of swine influenza virus for ferrets.

Method Used in Inoculating Ferrets Intranasally.—In the first attempt to infect a ferret, so much difficulty was encountered in introducing the inoculum intranasally, due to the animal's extremely effective sneeze reflex, that in most of the subsequent work the animals were lightly etherized prior to inoculation. This anesthetization was accomplished by using a museum jar 16 cm. in diameter and 28 cm. deep, in the bottom of which, under a wire mesh screen, was a piece of ether-soaked cotton. Ferrets to be anesthetized were placed in the jar, and the jar covered with a lid. The animals were carefully observed and as soon as they had dropped to the bottom of the jar they were removed for inoculation. This was now easily accomplished by using a 2 cc. record syringe without needle, applying the blunt tip of the syringe to the external nares and injecting a small amount of the infectious suspension each time the animal inspired until the desired dosage had been administered. Recovery from the anesthetic was prompt and accompanied usually by violent sneezing on the part of the inoculated ferret.

Inoculation of anesthetized ferrets in the manner described above has been adequately controlled, using either non-infectious suspensions or neutral serumvirus mixtures as the test inocula, and in no instance has any clinical evidence of

illness or pathological alteration detectable at autopsy been observed. These control experiments indicated that neither the process of anesthetization nor the introduction of fluid in 1 to 2 cc. amounts into the noses of anesthetized ferrets induced any clinical or pathological change which could be confused with the disease resulting from similar inoculation with infectious material of swine influenza. As will be described in more detail later, however, the severity and extent of the disease produced in this manner were greater than in animals infected without the aid of an anesthetic.

#### The Production of Discase in Ferrets by Intranasal Inoculation with Infectious Material from Cases of Swine Influenza

Four attempts to infect ferrets with four different samples of infectious material from experimental cases of swine influenza have all been successful. For these experiments 2 field strains of the disease were used: Strain 15, obtained from Iowa in 1930, and Strain 19, obtained from Iowa in 1933. Furthermore, the disease so produced proved readily transmissible in series through ferrets. One series has at present reached its 16th ferret passage. In passing from ferret to ferret the usual procedure has been to prepare an approximately 5 per cent suspension of pathological lung, or pathological lung plus bronchial exudate, from a ferret killed on the 3rd or 4th day following infection and to use 1 to 2 cc. of the supernatant fluid from this suspension in inoculating the next ferret intranasally. Where it has been necessary to delay infecting this animal the pathological lung from the ferret of the preceding passage has been stored, as chunks, in 50 per cent glycerol in the refrigerator. Material stored in this way for as long as 75 days proved fully infectious when tested.

#### Clinical Picture of the Disease in Ferrets

The clinical picture exhibited by the ferrets in the present experiments was similar to that described by Smith, Andrewes, and Laidlaw (1). Differences observed are attributable to a more extensive and severe pathological alteration in the respiratory tracts of our ferrets, the result possibly of two variations in technique. First, most of our ferrets were anesthetized prior to inoculation; and second, lung was used instead of turbinates as a source of infectious agent. It is also possible that American ferrets are more highly susceptible to infection.

The manner in which the disease progresses, in ferrets inoculated while anesthetized, is fairly regular. The period of incubation varies from 24 to 48 hours. An elevation of temperature, some inappetence, and mild depression are the first evidences of illness to be observed The first temperature rise is sharp, the fever usually reaching 41°C. and occasionally going as high as 41.6-42°C. By the following day the temperature has dropped from 1-2°C., food is refused, depression is more marked, and the catarrhal symptoms have appeared. On the 3rd day of illness, the temperature is usually up to fever level again, and the animal appears ill, lies listlessly in its cage, its fur is roughened, there is usually considerable nasal exudate, the eyes are watery or matted shut, sneezing and coughing paroxysms may be observed, and the respiratory rate is increased. Breathing may take on a jerking character suggestive of the thumping type of respiration seen in pigs with swine influenza (2). The 4th day usually finds the temperature down again and symptoms of respiratory embarrassment increased. Expiration may be accompanied by whining; sometimes the animals appear slightly cyanotic. Only 5 severely ill animals have been observed beyond the 4th day of illness. Of these, 3 died, one on the 5th, one on the 6th, and the other on the 8th day following infection. Their temperatures became subnormal, dropping to 34.6°, 35.9°, and 34.1°C. the day before death, cyanosis deepened, the respiratory rate was markedly increased, prostration became extreme, and breathing was accompanied by moan-The remaining 2 survived, after undergoing a severe illness which became progressively worse until the 7th day after infection when both animals were cyanotic, appeared moribund, and exhibited subnormal temperatures. However, on the 8th day they showed marked improvement and began a rapid and complete

While the general course of the disease was fairly regular as outlined above, individual symptoms were frequently inconstant. The catarrhal symptoms were in some cases mild, while in others they were so extreme that practically complete nasal obstruction resulted The temperature reaction, while in the majority of cases diphasic, was not invariably so; in some animals, after a primary rather sharp rise, it declined and became subnormal as the animal became more ill; in others it rose and remained elevated throughout 3 or 4 days that the animal was under observation. Rarely there was no temperature response to infection in spite of the fact that the animal became ill and was typical at autopsy. Depression, inappetence, and a general appearance of severe illness, together with evidence of respiratory embarrassment also varied in degree in individual cases, but were usually

The clinical picture presented by ferrets that had been inoculated without the aid of an anesthetic was qualitatively similar to that just described, but the animals were more mildly ill and showed no symptoms of serious pulmonary involvement. No ferret inoculated without anesthetizing appeared seriously ill when sacrificed on the 3rd or 4th day following infection and one such animal whose disease was allowed to run its course made an uneventful recovery after being mildly ill for a

#### Pathology of the Discase in Ferrets

In addition to showing the pathological alterations observed by Smith, Andrewes, and Laidlaw in the nasal passages (1), the ferrets in these experiments have exhibited marked and extensive lesions throughout the whole of the respiratory tract. Ferrets infected while anesthetized show, when autopsied on the 3rd or 4th day of illness, the following picture. The trachea and bronchi contain a thick, tenacious, glassy, white mucous exudate, which is usually profuse and frequently foams out when the trachea is cut open for culturing. The lungs are extensively affected and their involvement is of a lobar character. The lobes affected in the order of frequency are as follows; right diaphragmatic, azygos, right cardiac, left diaphragmatic, right apical, and left apical. Usually only the first three lobes mentioned are affected but occasionally an animal comes to autopsy showing a five lobe involvement and in the 3 fatal cases all six lobes were affected (Fig. 2). The involved lobes are deep bluish red in color; they appear distended and larger than normal and do not collapse when left standing, after removal from the animal, as do the uninvolved lobes. They have a gelatinous appearance in the gross and present a smooth, tense, and glistening pleural surface. When such lobes are cut across there is, as a rule, a rather copious outflowing of a frothy serosanguineous The cut bronchioles protrude and exude a slimy white mucous exudate. The lymph nodes at the hilum of the lung may be prominent and edematous but this is not a constant picture. Figs. 1 and 2 illustrate the gross appearance of the pathological lungs.

The white spots on the lung surface, described by Dunkin and Laidlaw (3) in their distemper-infected ferrets, have been seen in about one-third of the animals coming to autopsy. Since similar spots have been seen by the writer in apparently normal ferrets they are not considered significant with respect to the present experiments.

Histologically the trachea and bronchi are found to contain a variable exudate comprised usually of round cells, polymorphonuclear leucocytes, and desquamated epithelial cells imbedded in a pink-staining granular ground substance. The cilia lining the trachea and bronchi are usually either gone or badly matted together. The lining epithelium is, in places, fragmented and partially desquamated. Leucocytes are frequently to be seen passing out through the epithelium. Blood vessels in the submucosa are often dilated and packed with red blood cells. In some sections the smaller bronchi are cuffed by infiltrating round cells.

In the lung itself the histopathology is essentially that of an edematous pneumonia. The alveoli are, for the most part, distended and contain a few cells in addition to much coagulated plasma and some strands of fibrin. The majority of these cells are large with round nuclei and a relatively abundant pale-staining cytoplasm. Polymorphonuclear leucocytes and red blood cells are present in variable numbers. The leucocytes are usually most abundant in the alveoli adjacent to terminal bronchioles. The alveolar walls are greatly thickened due partly to the presence of dilated red cell-packed capillaries and partly to infiltration

with round cells and polymorphonuclear leucocytes. In addition to this there appears to be some actual proliferation of alveolar epithelium. of inflammatory cells in the alveolar walls is striking compared with the relativelysmaller numbers in the alveoli themselves. Polymorphonuclear leucocytes are more numerous in sections from lungs in which a bacterial invader has been present; in The large number such cases they not only infiltrate the alveolar walls but also lie free in the alveoli, sometimes in large numbers. In bacteriologically sterile lungs the leucocytes are present largely as cells infiltrating the alveolar walls. The histopathology of influenza in ferrets is similar, in many respects, to that of the disease in swine, especially to that seen in the more severe cases (2).

The pathological picture just described is that seen in ferrets infected while anesthetized. In animals inoculated intranasally without the aid of an anesthetic the picture is similar, but less extensive. Fewer lobes of the lung are involved and the distribution of the pulmonary lesions is more patchy and less lobar in character. One ferret inoculated without anesthetizing exhibited a definite but mild illness and at autopsy showed no pulmonary lesions. This animal's nasal passages were inflamed and the turbinates covered with a scant seromucous

## Bacteriology and Filtration Experiments

A filtrable virus and the organism H. influenzae suis have been shown to be etiologically essential to the production of influenza in swine (4, 5). Bacteriological study of the respiratory tracts of ferrets sick following intranasal inoculation with infectious material of swine influenza did not suggest that any single bacterial component played a rôle in the ferret disease. Frequently the tracheal exudate and involved lobes of the lung were sterile. When an organism was encountered it was seldom the same as that found in the preceding ferret passage. No single bacterial form was encountered with any degree of constancy. Bacteria were present in the lungs of all 3 fatal cases. Since the suspensions used in infecting 2 of the 3 fatal cases had been prepared from bacteriologically sterile ferret lungs, it seems likely that the organisms encountered in the lungs of the animals that died were of ferret origin and had been present in the animals' respiratory tracts prior to infection. Whether the presence of such bacteria in the respiratory tracts of infected animals contributed to the severity of their disease is not known. II. influenzae suis, present in the infectious material of swine origin used in infecting the first ferrets in each set of ferret serial passages, did not establish itself in the ferret respiratory tract, and was not isolated even from the first passage ferrets.

The component of the swine influenza etiological complex causing disease in ferrets was thus, presumably, the swine influenza virus. Two attempts to infect ferrets with bacteriologically sterile Berkefeld N filtrates of known infectious material have resulted positively, producing a disease which, while somewhat milder than that induced by the unfiltered suspensions, was nevertheless qualitatively typical. At autopsy both filtrate-infected ferrets exhibited characteristic pulmonary lesions. The tracheal exudates and pathological portions of the lungs of both animals proved bacteriologically sterile. Passage from one of the filtrate-infected ferrets resulted in the characteristically severe disease in the next serially infected animal. This indicated that the milder character of the illness in the filtrate-infected animals had been due only to a dilution of virus during the process of filtration and not to the loss of some etiologically essential component other than virus.

Infectious material (lung) from sick ferrets after storage in 50 per cent glycerol at refrigerator temperature for periods of 26, 30, 53, and 75 days, has proved to be fully infectious for ferrets. This ability of the infectious agent to preserve well in glycerol, together with its ready filtrability and its transmissibility in series through ferrets indicates that it is a virus.

Failure of the Virus to Infect Ferrets When Administered Subcutaneously.—Ferrets, anesthetized with ether and then inoculated subcutaneously with 2 cc. amounts of virus known to be active, exhibited no clinical evidence of illness and were completely negative when autopsied 6 days following inoculation. This is in accord with the experience of Smith, Andrewes, and Laidlaw and indicates a tropism of the virus for respiratory tract tissues similar to that exhibited by the swine influenza virus in swine (6).

The experiments thus far described indicate that there is a virus present in infectious material from cases of swine influenza that is pathogenic for and transmissible in series through ferrets. They confirm the earlier experiments of Smith, Andrewes, and Laidlaw (1) on this point completely. The remainder of the experiments to be described were conducted in the attempt to establish whether this virus is actually the swine influenza virus.

## The Infection of Swine with Ferret-Passaged Virus

If the virus that produced disease in ferrets was the swine influenza virus it would be expected to induce swine influenza when mixed with H. influenzae suis and administered intransally to swine (4), unless ferret passage had attenuated it for its natural host. 2nd, 6th, 10th, 12th, and 16th ferret passage virus mixed with H. influenzae suis has been administered intranasally to swine. All 5 pigs inoculated developed perfectly characteristic swine influenza indistinguishable in any respect from that induced by similar inoculation with virus of swine origin. No lengthening of the incubation period, decrease in clinical severity, nor diminution in the extent of the pathological alterations encountered at autopsy was observed in these 5 swine. The disease induced in hogs by the ferret virus mixed with H. influenzae suis proved to be further transmissible in series through swine either by pen contact or intranasal inoculation. One pig inoculated with 6th passage ferret virus without the addition of H. influenzae suis developed only the mild filtrate disease (4).

The above data are evidence that the virus, responsible for the disease in ferrets, was actually the swine influenza virus.

# The Neutralization of Virus from Swine and Ferrets by Ferret and Swine

To obtain further evidence that the virus causing disease in ferrets was identical with the swine influenza virus two separate sets of crossneutralization tests in the two susceptible hosts were conducted.

The results of the experiments shown in Table I may be briefly summarized as follows. Serum from swine recovered from swine influenza was capable of neutralizing the ferret virus when the serum-virus mixtures were tested for neutrality by intranasal inoculation into either swine or ferrets. Serum from ferrets recovered from the ferret disease was capable of neutralizing swine influenza virus when the serum-virus mixtures were tested for neutrality by intranasal inoculation into either swine or ferrets.

In conducting the neutralization tests, the ferret virus used was either fresh or glycerolated pathological lung from ferrets killed on the 3rd or 4th day following infection, while the swine influenza virus used was either fresh or glycerolated pathological lung and bronchial lymph nodes from swine killed on the 3rd or 4th ay of a typical attack of swine influenza. Both were prepared as approximately

The Neutralization of Virus from Swine and Ferrets by Ferret and Swine Influenza Convalescent Scrum TABLE I

valescent Serum	Remarks		Susceptible to infection when tested later .  Died 6th day		Autopsy negative when killed on 4th day	killed on 3rd day	Autopsy negative when	killed on 6th day Autopsy typical when	killed on 4th day	Autopsy typical when killed on 6th day	Autopsy typical when		Susceptible to infection	when tested later Autopsy typical when	killed on 4th day
er er er und Swine Influenza Convalescent Serum	Result	No illinois	Severe illness	77.	Severe illness		No illness	Severe illness		No illness for 3 days then typical swine	Influenza Typical swine influ-	enza	No illness	Typical swine influ-	ciiga
nun acus e Co	Source and type of serum and amount	Swine influenza conva-	lescent serum—1 cc. Normal swine serum—	Swine influence connection	cent serum—1 cc. Normal swine serum—	1 cc.	Swine influenza convales-	Normal swine serum—		Jescent serum—10 cc.	Normal swine serum—	Surino in G.	cent serum—10 cc.	Normal swine serum—10 cc.	
	H. influenzae suis mixed with		°N		No		No			Yes			Yes		
	Source of virus and amount	Ferret 2 (2nd passage) —1 cc. Ferret 12 (4th passage) —1 cc.			Ferret 24 (10th pas- sage)—0.66 cc.			Ferret 24 (10th passage)—5 cc			Ferret 26 (12th pas-	3age)—1 cc.			
	Animal No.	Ferret 6	Ferret 5	Ferret 14	Ferret 13	Rorrot 22	Formet 22	1 61161 23	Swine 1441		Swine 1442	Swine 1429	Swine 1428		
	Experi- ment No.	•	7	,	7		ю			71		t.	ი		

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Animal resisted infection when tested later killed on 4th day  Autopsy bypical when killed on 5th day killed on 5th day killed on 4th day killed on 4th day killed on 4th day killed on 4th day when tested later	killed on 4th day
	killed on 4th day
t se- No illness    Severe illness   Severe illness     Severe illness   No illness     Typical swine influcation     Typical	
Ferret convalescent se-   No illness   Normal ferret serum   Severe illness   Ferret convalescent se-   No illness   Normal ferret serum   Typical swine influ-   Severe convalescent se-   No illness   Sec.   No illness   Sec.   No illness   Sec.   No illness   Sec.   No illness   Severet serum   Typical swine influ-   Severet serum   Severet serum   Severet serum   Typical swine influ-   Severet serum   Typical swine influ-   Severet serum   Se	
Perret convalescent  Normal ferret serum- 1 cc. Ferret convalescent sc. Normal ferret serum- 5 cc. Perret convalescent sc. Normal ferret serum- 7 cc. Normal ferret serum- 7 cc.	
rc. Ncs.	
Swine 1375—1 cc.  3   Swine 1376—3 cc.	
Ferret 7   Swine 1363   Swine 1445   Swine 1448   Swine	
, , , , , , , , , , , , , , , , , , ,	

5 per cent suspensions in physiological saline and the supernatant fluid from such suspensions used as the source of virus. The swine virus used had at no time been passed through ferrets and the swine influenza convalescent serum had been obtained from pigs infected with virus that had at no time been passaged through ferrets. A small quantity of a culture of *H. influenzae suis* was added to each of the serum-virus mixtures to be tested in swine, since, in this species, both this organism and the virus are etiologically essential to the production of the typical disease (4). The serum-virus mixtures were, in all instances, incubated for 30 minutes at 37°C. and then stored for 2 hours in the refrigerator prior to intranasal inoculation into the test animals. In the cases of the swine inoculations, the culture of *H. influenzae suis* was added to the serum-virus mixture after the preliminary period of incubation and storage and immediately prior to inoculation into the test swine. All ferrets used in these neutralization experiments were anesthetized with ether to facilitate inoculation and to enhance the severity of the disease.

Experiments 4, 5, and 6 recorded in Table I require some comment. In Experiment 4, Swine 1441, which received a mixture of 5 cc. of ferret virus suspension, 10 cc. of swine convalescent serum, and 1 cc. of a suspension of a culture of H. influenzae suis, was not completely protected and developed influenza after an incubation period of 3 days, as compared with a 24 hour incubation period for its control, Swine 1442. It was evident, from the lengthened incubation period, that the virus had been partially neutralized. Using a smaller amount of virus in Experiment 5, ferret virus was completely neutralized by swine influenza convalescent serum. In Experiment 6, Ferret 7, inoculated intranasally with a mixture of swine influenza virus and ferret convalescent serum, developed no illness, while its control, Ferret 8, came down typically. Ferret 7 proved immune to reinfection, however, when tested later. In view of the fact that not one of the 35 ferrets inoculated intranasally with swine influenza virus during the course of these experiments has resisted infection, it seems likely that, in the case of Ferret 7, the serum-virus mixture it received was neutral enough to prevent a clinically recognizable infection, but not to prevent immunization.

#### DISCUSSION

The experimental data presented in the last two sections of this paper are such as to indicate conclusively that the virus from swinc influenza infectious material, capable of producing disease in ferrets, is actually the swine influenza virus. The virus has been observed to produce a definite and constant disease picture in ferrets for 16 serial passages and when, mixed with *H. influenzae suis*, it has been tested in swine at the end of the series as well as during the course of the passages, it has regularly produced characteristic swine influenza.

The neutralization of ferret-passaged virus by swine influenza convalescent serum and of swine influenza virus by ferret convalescent serum confirms the identity of the virus producing disease in ferrets,

The disease in ferrets described in the present paper was clinically more severe and pathologically more extensive than the swine influenza virus infection in ferrets described by Smith, Andrewes, and Laidlaw (1). It was suggested earlier that this greater severity may have been contributed to by three factors; a possible greater susceptibility of American ferrets to infection, the use of lung instead of turbinates as a source of virus, and the anesthetization of the animals to facilitate inoculation. The rôle played by the first factor in yielding a more severe disease cannot be determined from the data given in the present experiments. That it is probably of minor significance, however, is indicated by the fact that Smith, Andrewes, and Laidlaw have stated that they too obtain pulmonary lesions, similar to those described in the present paper, in English ferrets inoculated with swine influenza virus under ether anesthesia. The importance of the second factor is doubtful because in a single experiment in which turbinates as well as lung from a sick ferret were used to inoculate ferrets, under ether anesthesia, characteristically severe disease resulted in both animals. The illness developing in the ferret infected with virus from the turbinates was just as marked as that shown by the ferret infected with virus from the lung, and extensive pulmonary lesions were encountered in both animals at autopsy. This would indicate that substitution of lung for turbinates as a source of virus did not account for the greater severity of the ferret disease seen in the present experiments as compared with that described by the British investigators (1). This leaves the rôle played by the third factor, anesthetization with ether, for consideration. It has been shown in the present experiments that anesthetization of ferrets prior to their inoculation with virus definitely increases the severity of the resulting disease. Two explanations for this are suggested: first, that the irritation of the respiratory tract by ether favors the establishment of the virus, and second, that due to inhibition of the sneeze reflex,

by the anesthetic, a much larger dose of virus is given and it is introduced more deeply into the respiratory tract. It seems probable from the evidence available that the second explanation is most applicable and that swine influenza in ferrets represents a purely local infection in which lesions are produced in the respiratory tract only where the virus is introduced mechanically.

The disease that Smith, Andrewes, and Laidlaw have described unquestionably more nearly represents the natural infection of ferrets with swine influenza virus than does the disease produced by the methods here described. The use of anesthetization with ether in infecting ferrets with swine influenza virus, however, presents the advantages of facilitating inoculation and giving a more definite, constant, and easily recognizable disease with which to work.

Experiments designed to determine whether or not swine influenza virus was capable of transferring from ferret to ferret by contact have so far yielded inconclusive results. However, in all of the experiments recorded in this paper the isolation of animals was rigid enough to prevent accidental infections by contact.

#### SUMMARY

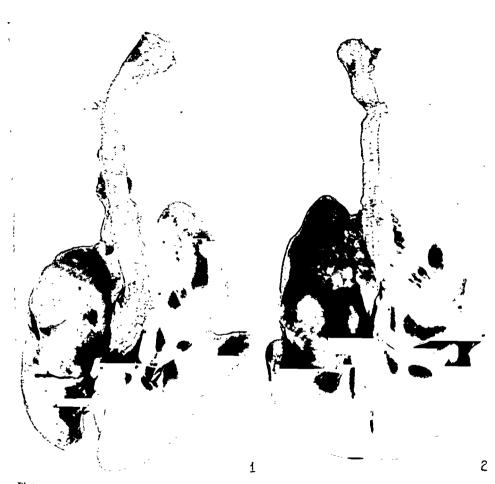
The experiments described confirm the earlier observation of Smith, Andrewes, and Laidlaw that the swine influenza virus is pathogenic for ferrets when administered intranasally. A disease that is clinically more severe and pathologically more extensive than that described by the above workers is obtained if inoculation with the virus is performed under ether anesthesia. Animals infected in this way show at autopsy an edematous type of pneumonia of lobar distribution which may terminate fatally. The virus maintains its pathogenicity for ferrets when stored in 50 per cent glycerol at refrigerator temperature for as long as 75 days. After serial passage through 16 ferrets the virus is still capable of inducing swine influenza when mixed with H. influenzae suis and administered intranasally to swine. Ferret passage causes no apparent attenuation of the virus for swine. Serum from pigs recovered from swine influenza is capable of neutralizing the ferret-passaged virus for either swine or ferrets. Likewise serum from recovered ferrets neutralizes the swine influenza virus for either ferrets or swine.

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- 3. Dunkin, G. W., and Laidlaw, P. P., J. Comp. Path. and Therap., 1926, 39, 201. 5. Lewis, P. A., and Shope, R. E., J. Exp. Med., 1931, 54, 361.
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- FIG. 1. Lung of Ferret 23 infected intranasally, under ether anesthesia, with an EXPLANATION OF PLATE 3 unfiltered but bacteriologically sterile suspension of ferret lung swine influenza virus (10th ferret passage virus). Animal sacrificed on 4th day following infection. There is an edematous pneumonia of all three lobes on the right side. Both lobes on the left side show earlier lesions.
- Fig. 2. Lung of Ferret 28 infected intranasally, under ether anesthesia, with an unfiltered suspension of ferret lung swine influenza virus (12th ferret passage virus). Animal died on 5th day following infection. There is an edematous pneumonia of all lobes of the lung.





Photographed by J. A. Carlile



# THE FLOW AND COMPOSITION OF LYMPH IN RELATION

 $_{
m By~A.~A.~WEECH,~M.D.,~E.}$  GOETTSCH, M.D., and E. B. REEVES (From the Department of Diseases of Children of Columbia University College of

(Received for publication, January 19, 1934)

Present day theories of the pathogenesis of edema assign great importance to the blood proteins in maintaining normal fluid distribution between blood and tissue spaces. The original observations of Starling (1) have been confirmed and supported by the work of Epstein (2), Krogh (3), Schade and Claussen (4), Peters and his associates (5), Leiter (6), Barker and Kirk (7), and a host of others. A wealth of evidence exists to support the belief that the plasma proteins, because of the colloidal size of their molecules, are unable to diffuse to any great extent across the capillary wall and, therefore, exert an osmotic pressure which serves to prevent excess accumulation of fluid in the tissues. Evidence for the impermeability of the capillary to protein has been necessarily indirect as tissue fluid normally exists in such small amounts that it cannot be obtained for analysis. It has long been known, however, that lymph from various regions does contain protein. Within the last few years, Drinker and Field (8) have shown repeatedly that lymph from vessels which drain the subcutaneous tissues is no exception in this respect and have given reasons for their belief that this lymph possesses "an approximate degree of identity" with tissue fluid in the corresponding region. From the quantitative standpoint, at least, proof of this contention will require modification of the Starling theory.

The experiments to be described in this paper were designed to test directly the relative compositions of lymph and one form of tissue fluid. As originally conceived the plan of study was simple. Edema was to be produced in dogs, either rapidly by plasmapheresis or slowl by protein starvation, and the composition of edema fluid compare directly with that of lymph collected from one of the lymphatic trunks draining the edematous region. This purpose has been accomplished. In addition phenomena have been encountered which on the one hand have brought a clearer understanding of the circulation through the tissue spaces and on the other have marked out features concerning which existing knowledge is still inadequate. The findings collectively form the basis of this communication.

To avoid confusion in terms it is necessary at the outset to define briefly the sense in which several words and phrases are to be employed. The word lymph will be used only when referring to the fluid content of the lymphatic vessels and not as synonymous with tissue fluid as has been done so frequently in the past. Capillary filtration, capillary filtrate, and capillary reabsorption will be employed in the exact sense which the words imply. The terms tissue fluid, interstitial fluid, and intercellular fluid will be used interchangeably to refer to the fluid which is present in the interstitial spaces and which is outside the walls of both blood capillaries and lymph capillaries. A distinction will be made between the phrases rate of lymph flow and lymph formation or lymph production. The former will apply to the volume of lymph delivered by a given lymphatic vessel in a unit time and the latter two to the process of generating fluid which under certain circumstances can enter the lymphatics and give rise to lymph flow.

#### Methods

The dogs used were of mongrel breeds and varied in weight from 15 to 26 kilos. Lymph was obtained exclusively from vessels of the lower leg into which cannulas were inserted some inches above the ankle but well below the knee or elbow. Lymph so obtained has not undergone any theoretical alteration by filtration through lymph nodes and comes in large part from regions liable to be affected with subcutaneous edema. In earlier experiments the small operation necessary for locating and cannulizing a lymphatic trunk was performed under general anesthesia (either nembutal or ether); in later experiments local anesthesia with novocaine was found quite sufficient. Lymph flow was stimulated either by gentle massage of the foot and ankle combined with passive motion of the extremity or by allowing the animal to walk or run about in the manner described by White, Field, and Drinker (9). The rate of flow was determined by measuring the volume of lymph entering the cannula in an arbitrary time.

The methods of producing edema by plasmapheresis and by the limitation of protein in the diet have been described in a previous paper (10). Edema fluid

A slight modification has been made in the composition of the diet, that is, cod liver oil, 10 cc. daily, has been substituted for the butter fat. Animals maintained on the modified diet have remained in better health than those on the original diet and have exhibited fewer skin lesions. The cod liver oil was supplied

was obtained by puncturing with a needle into an edematous region and applying was obtained by puncturing with a needle into an edematous region and appropriate gentle pressure to the surrounding tissues. Most frequently this region was the genue pressure to the surrounding assues. Prost requently this region was the loose tissue about the Achilles tendon of a hind leg. In several instances samples of ascitic fluid were obtained by puncture of the peritoneum. Samples of edema of ascure many were obtained by puncture of the peritoneum. Samples of column fluid and lymph were analyzed for total protein by a micro-Kjeldahl procedure nund and lymph were analyzed for total protein by a micro-lajouani protein which has been previously described (10). Whenever possible, samples of from which has been previously described (10). Whenever possible, samples of Holl of the Control of t minations rarely exceeded 0.02 gm. per cent and was often less than 0.01 gm. per minations rately exceeded 0.02 gm. per cent and was often ress than v.o. 6m. per cent. Non-protein nitrogen was determined separately whenever the volume of sample permitted; in a few instances the figure obtained by analyzing blood serum collected at the same time was used in the calculation of protein from total deter-Lymph Flow in Normal Dogs mined nitrogen.

The data collected in these experiments are given in Table I.

The rates of lymph flow are expressed as cubic centimeters produced by the entire foot in 10 minutes, the calculation being the same as that employed by White, Field, and Drinker (9). It is based on the observation that a concentration of vessels occurs at the wrist and ankle, so that in both cases "there are three, tion of vessels occurs at the winst and anxie, so that it both care is no detectable difference or at the very most four, draining trunks." As there is no detectable difference or at the very most rour, manning trains. The there is no detectable difference in size one may assume that the flow through all is equal. A fair approximation of the flow of lymph from a part is therefore secured by multiplying the flow from

The values recorded for each animal exhibit considerable variation. To appraise them properly several observations are pertinent. Dura single vessel by three.2 ing periods of anesthesia or at other times of complete physical inactivity lymph flow ceases entirely. If a single movement occurs, or if the extremity is stroked, lymph at once rises in the cannula and may continue to rise at a declining speed for some seconds subsequently. Within a brief time the flow again ceases. The values recorded in Table I for sitting animals are, therefore, not a measure of lymph flow during physical inactivity but indicate merely that the sitting dog is rarely completely at rest. When, after a period of rest, lymph flow is stimulated by regular massage or motion, the first lymph always

2 For those who may think that this method of expressing the rates of lymph flow involves too many assumptions, it is possible to regard the figures which express the volume of lymph draining from the entire foot in 10 minutes as representing more accurately the volume delivered by a single lymphatic trunk in 30 minutes.

TABLE I
Rate of Lymph Flow in Six Normal Dogs

=====	<del></del>	<del></del>							
Time	Anesthetic	Activity	Rate	Time	Anesthetic	Activity	Rate		
	$\mathbf{D}_{0}$	g 10-00			n	og 1-31	<del></del>		
		fore leg)				hind leg)			
-	<del></del>	·	<del></del>	-	1 (.076	1	<del></del>		
2:15		J	cc.		1	1	cc.		
2:15	N	Walking	0.9	11:25	L	Massage	-		
	N	Running	1.2	12:25	L	Walking	1.8		
3:15	N	Sitting	0.6	12:55	L	Walking	1.6		
3:45	N	Walking	0.7	1:25	L	Walking	1.6		
4:15	N	Walking	0.7	2:05	L	Massage	-*		
4:45	N	Walking	0.7	2:45	L	Walking	2.5		
5:15	N	Walking	1.2	3:15	L	Walking	2.4		
		og 9-2		Dog 8-40					
	(left )	hind leg)		(right hind leg)					
12:45	L	Massage	2.2	10:45	L	Massage	4.4		
1:00	L	Massage	1.0	11:15	L	Walking	1.6		
1:15	L	Massage	1.2	11:45	Ľ	Walking	1.7		
2:00	L	Massage	1.0	12:15	L	Walking	1.4		
2:23	L	Walking	0.1	12:45	L	Running	2.5		
2:50	L	Walking	2.9	1:53	L	Massage	2.6		
3:10	L	Walking	2.7	2:15	L	Walking	2.1		
3:30	L	Walking	2.7	2:45	L	Walking	2.3		
	Do	og 5-8	<u>!</u>	3:15	L	Walking	1.3		
		fore leg)	i		Do	g 9-1			
2:00	E	Massage	11.7	(left hind leg)					
3:15	E	Massage	9.5	2:45	L	Walking	0.8		
4:00	N	Walking	5.2	3:15	L	Running	0.8		
4:30	N	Running	7.3	3:45	L	Walking	0.6		
5:00	N	Sitting	0.5	5.10		,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	- • •		
5:30	N	Walking	4.8	1	1	ł			
	1	.,		<u> </u>	1				

Lymph flow is expressed as total lymph from foot in 10 minutes. Lymph from single lymphatic trunk is multiplied by 3 to obtain total lymph. Collections were continuous throughout successive periods except as noted. Time, as entered in the table, refers to the median time during a period of collection. Thus, the rate of flow at 2:45 p.m. may be calculated from the volume collected between 2:30 and 3:00 p.m., or from the volume collected between 2:40 and 2:50 p.m., etc. Dog 8-40 rested between 1:00 and 1:45 p.m. while receiving 500 cc. of physiologic salt solution intravenously. Dog 9-2 rested between 2:40 and 3:10 p.m. while receiving 650 cc. of Locke's solution intravenously and Dog 1-31 rested between 1:40 and 2:05 p.m. while receiving 500 cc. of Locke's solution intravenously.

The following abbreviations are used in this and subsequent tables. E, ether anesthesia; B, nembutal [sodium-ethyl (*l*-methyl butyl) barbiturate] anesthesia; N, normal state after recovery from ether anesthesia; and L, local novocaine anesthesia.

\* Observations during these intervals are omitted as the conditions of collection and measurement were altered. See Table II.

flows at a relatively rapid rate. As the stimulation is continued the rate decreases and finally attains approximately constant values. The maximum rates in Table I were measured immediately after periods of rest and were calculated from the volumes of lymph collected in the first 10 or 15 minutes. Table II records an experiment in which after a period of rest lymph flow was measured over a number of 15 second intervals. The short interval has accentuated the rapidity of the initial flow. A relatively constant rapid rate was main-

TABLE II

Rate of Lymph Flow during Successive Time Intervals Following a Period of Rest
Dog 1-31. Left hind leg. Local anesthesia.

Activity	Time interval	Volume of lymph collected	Rate of flow from foot		
		α.	cc. per 10 min.		
None	25 min.	0	0		
Massage	15 sec.	0.30	36.0		
Massage	12 sec.	0.26	39.0		
Massage	15 sec.	0.37	44.4		
Massage	15 sec.	0.27	32.4		
Massage	15 sec.	0.30	36.0		
Massage	15 sec.	0.20	24.0		
Massage	15 sec.	0.15	18.0		
Massage	30 sec.	0.20	12.0		
Massage	30 sec.	0.25	15.0		
Massage	30 sec.	0.30	18.0		
Massage	30 sec.	0.10	6.0		
Massage	1 min.	0.20	6.0		
Massage	2 min.	0.20	3.0		
Walking	30 min.	2.50	2.5		
Walking	30 min.	2.40	2.4		

tained for 75 seconds only; thereafter, it declined progressively. In this case the initial rate of flow, approximately 38 cc. per 10 minutes, permits an estimate of the carrying capacity of the vessels and it is clear that this capacity greatly exceeds the normal rate of continuous flow. The fact will become significant when the findings with edematous dogs are considered. It is believed that the temporarily rapid initial flow depends on the quantity of extracapillary and intercellular fluid which has accumulated during the preceding period of

inactivity. With the commencement of activity this fluid is drawn at once into the lymphatics. In contrast the rates recorded after a constant flow has been established must measure the formation of fresh lymph. Such rates have regularly exhibited minor fluctuations but in general have been close to the minimum rates observed during activity. On this basis the rate of lymph production in the foot of a normal active dog can be estimated as  $1.45 \pm 1.20^3$  cc. per 10 minutes. The figure represents the average of the minimum rates of flow in six dogs of this series and in eight dogs studied by White, Field, and Drinker (9).4

The data in Table I confirm the observation of White and his associates (9) that lymph flow varies with the degree of activity. The variation between flows produced by moderate and excessive activity (walking and running) is, however, small in comparison with the temporarily increased rate which follows complete rest.

#### Lymph Flow in Edematous Dogs

When edema is produced in dogs either by plasmapheresis or by dietary restriction, it rarely happens that all four extremities are involved simultaneously. For this reason it has been possible to measure lymph flow in edematous dogs from non-edematous extremities as well as from edematous extremities. With edematous extremities lymphatics have always been selected for cannulization which included the edematous part in the region drained. Because the toes and paws are more swollen in nutritional edema than in plasmapheresis edema, lymph from protein-fasted dogs drains more generally from edematous regions than that from animals treated by plasmapheresis. The rate of flow from seven edematous extremities in six dogs is shown in Table III. The average maximum rate of flow was 19.3 cc. in 10 minutes, a higher figure than was recorded with any normal animal. Individual measurements, however, exhibited much variation which in a rough way could be correlated with the degree of edema present. For example, the left hind leg of Dog 2-3 presented a mild edema and

<sup>&</sup>lt;sup>3</sup> Standard deviation of the distribution.

<sup>&</sup>lt;sup>4</sup> For the discussion which follows the reader should note that in speaking of the rate of lymph formation we shall be referring to data secured through a study of the minimum rates of lymph flow.

Rate of Lymph Flow in Edematous Dogs from Extremities Which Exhibi na

Aule of T.	-172
TABLE III  Time   Anesthetic   Activity   Rate   Time   Dog 5-8	1
in Edematous De	
Time Anesthan Dogs from 1	Profuse to
Activis Activis	Alfemilies Which P
Rate	men Exhibited Es
Dog 5-8	Apacal
(right hind leg)	Activis 1
Tamid leg)	I Kata
4:15	Dog 2 2
4:30 L No.   CC.	(left hind leg)
1.30   1 Massage   1	leg)
2:33   r   Walking   27:0   11:4-	
5.0-   4   70   70   71.43	L Me Co.
	T Massage
3 0   12.40	
$D_{00} = 1.24$	L Wall-in 1.8
(right hind leg) 1:50	
(right hind leg) 1:50	
12:33   7	r   Walking   2.4
14.40   Alace	_   Walking   2.7
1:00   1   Marie   30.0	
	1)00.2.2
1:22   L   Walking   7.8	(right fore leg)
	Teg)
2.50   T   Walking   2.5    12.0-	L
3.00   r   Walking   2.7    12.22	L Massage 49.2
3.30   +   Massace   2.0    12	T 11255200 1 -2.2
L   W. v. Sc   2 5    12:45	T   -11assamo   -1.4
	r   Walking   -0.0
$D_{00}$ 0 : $U_{150}$	
100he 1 ·	1 172 - 16   2 0
	Walking 3.2
12:30 L Massage	
12   4   3   11 -	D07 9 40
12:50 L Massage 11.6	eft hind leg)
"20   T   Walking   "2   1	Tand leg)
1.45   r   Walking   1.0   4.45   L	1
2.13   T   Walking   U./    4 az   L	Massage
2.45   r   Walking   3.9    = 0   L	1-11355am
3:15   2   13:00   3:10	113552gg   4.0
3:45 L Walling 20	Walking   1.4
	-6 1 2 .
	10-
ngh	t hind leg)
Walking   3.4   3:00   - 1	- reg)
	Massage 14.2
In Dog 5	Walking 14.3
2.3 over 5-8, 9-1, and 4:20   L	Walking 14.3 Walking 7.1
L L	Walking 7.1
cived intravened was due to a coema resulted	Walking 5.4
In Dogs 5-8, 9-1, and 1-31 the edema resulted from plasma received intravenous injections of 500 cc. physiologic ween the 3:45 p	5.3
ween the a octween the same of 500 cc, phase alone	oge 5. In Doge
the side - in side n - Pursiologic - Die. D	ore in Doge

2-3, and 8-40 edema was due to protein starvation alone. Dogs 5 and 9-1 both ceived intravenous injections of 500 cc. physiologic salt solution; with the former resulted from plasmapheresis. In Dogs is occurred between the 3:40 p.m. and 4:20 p.m. periods and with the latter

the maximum rate was 3.9 cc. in 10 minutes, whereas the right fore leg was markedly swollen with edema and yielded lymph at an initial rate of nearly 50 cc. per 10 minutes. As in the normal animal, the initial rate of flow was invariably greater than that subsequently recorded, the decline being greatest when the initial rate was highest. The rapid decline from the initial rate indicates that even in the edematous animal the lymphatics are able to carry fluid from the tissues at a faster rate than newly formed fluid can accumulate. The maximum rates of flow are probably not greater than those in normal animals during the first seconds following a period of rest and merely indicate the presence of an increased accumulation of extracapillary fluid (edema fluid) which is able to maintain an accelerated flow for a longer space of time. Table IV presents the observed rates of lymph flow in three edematous dogs from five extremities in which palpable edema was not demonstrable. An average maximum rate of flow of only 2.90 cc. per 10 minutes again exemplifies the dependence of initial flow on the degree of edema.

The minimum rate of flow, which provides an estimate of the rate of lymph formation, is interesting in both groups. With edematous extremities the average minimum rate was 2.57 cc. per 10 minutes and with non-edematous extremities it was 0.72 cc. per 10 minutes. Although a real difference between the groups may exist, the result suggests that observations on the edematous extremities may not have continued long enough to furnish a record of the true minimum rates. In any case an average minimum rate for both groups together of 1.80 cc. per 10 minutes is not significantly different from that of 1.45 cc. per 10 minutes which was measured with normal dogs.

### Effect of Stimulating Lymph Flow on Edema

The conclusion reached in the preceding section that during initial activity (massage and passive motion or normal walking) lymph drains from an extremity more rapidly than new lymph can be formed and that this flow depends upon previously accumulated extracapil-

<sup>5</sup> When the amount of edema is considerable several hours may elapse before constant rates of flow are established at minimum levels. With animals weakened by prolonged restriction of protein in the diet or by frequently repeated plasmapheresis operations, long continued physical activity is often impossible.

Rate of Lymph Flow in Edematous Dogs from Extremities Which Were

In Edematous Dear 6
Non-Edematous Dogs from Extremities Which Were  Activity Activity
Anesthetic Activity A
I Pate II
Dog 9 on
TO TOP I
Dog 6 (right form)
12:30 B Massage (right fore leg)
1:00   B   Massage   2:00   B
1:15   Massage   7.7   2:15   Massage   4.
1:30   D   Massage   1:0   2:30   D   Massage   2.8
1:45   D   Massage   1:0   2:45   D   Massage   1:0
7:00   Alacon   2:2    2:00   D   3: 0   1
_   N   \
2:15   B   Massage   0.8   3:15   B   Massage   1.8   2:30   B   Massage   3.7
R   Massage   10   Massage
1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
1.45 D
2:00 D Alassage
B   Lassage   2 6   2:15   D   Massage   2:3
B   Massage   1 /   2.30   B   Massage   3.3
2:00   p   Massage   2:45   B   Massage   1.4
2:15   n   Massage   3:00   B   Mass
2:30   p   Massage   3:15   B   Mg   3:14
2:30 B Massage 0.6 3:15 B Massage 1.6  Massage 1.6
1126320
1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
(left fore leg)
3:15 L Walking
3:15 L Walking 3.2
Edema 41 Walking V.S
tempt was result of plasmaph. 7:45 L Walking 0.9
Edema the result of plasmapheresis. With Dog 5-8 after initial 3
oride in 500 cc. water. With Dog 5-8 after initial 3
reased. A mater. Within who own after initial?

the result of plasmapheresis. With Dog 5-8 after initial 3 collections attempt was made to stimulate lymph flow by giving by gavage 10 gm. sodium chloride in 500 cc. water. Within 12 minutes diarrhea resulted and lymph flow decreased. A subsequent gavage with 500 cc. physiologic salt solution and an intravenous injection of 500 cc. physiologic salt solution were not attended by significant variation in the rate of lymph flow. The increased flow at the 6:05 p.m. period followed a previous rest period of 20 minutes.

lary fluid, is verified in a striking way when one observes the effect of such activity on the edematous state. Repeatedly it has been observed (Dogs 5, 5-8, 9-1, 8-40) that swelling and evidence of edema slowly disappear during these periods. With extremities carrying a cannula in a lymphatic trunk the decrease in clinical edema has always been correlated closely with the declining rate of lymph flow. In these cases a precisely similar decrease has always been seen in non-operated extremities and the phenomenon can, therefore, bear no relation to the operative procedure. Under such circumstances it is impossible to escape the conviction that edema fluid, that is, interstitial fluid, is being drained from the tissues through lymphatic channels. Finally, it may be noted that when edema is eliminated in the manner described it has always reformed after a subsequent overnight rest period.

#### Protein in Lymph from Normal and Edematous Dogs

In a recent monograph Drinker and Field (8) have brought together the observations of numerous investigators which show that lymph collected from a variety of animals under different conditions and in different regions always contains an appreciable and sometimes a considerable quantity of protein. They have tabulated fifteen of their own observations concerning leg lymph obtained from quiescent dogs under sodium barbital anesthesia. This lymph exhibited a protein concentration (refractometer) which varied between 0.70 and 5.71 gm. per cent and averaged 1.84 gm. per cent. The authors believe that the higher concentrations exist for short periods only and point to the experience of White, Field, and Drinker (9) who measured the protein content of lymph from non-anesthetized dogs during periods of normal activity. "When constancy of protein was reached in eight dogs, the concentrations were from 1.52 to 0.5 per cent of protein."

The results of our investigation (Table V) are in substantial agreement with those reported by the above authors. Among twelve normal dogs protein concentrations varying between 0.45 and 3.45 and averaging 1.59 gm. per cent were encountered. From five of the dogs specimens were collected during nembutal anesthesia. These showed an average of 2.06 gm. per cent of protein as contrasted with a figure of 1.25 gm. per cent from seven non-anesthetized dogs. The highest concentration observed in the absence of anesthesia was 1.89 gm. per cent. Table VI presents the concentrations of protein in the lymph from ten edematous dogs. The values range from 0.01 to 0.69 gm. per cent, average 0.23 gm. per cent, and are considerably lower

TABLE V Proteins in Lymph and Serum of Normal Dogs

	Proleins in I	
Dog No.	Anesthetic Predominating Protein of lymph  Protein of lymph  Protein of lymph	
8-41 8-42 8-95 9-49 9-50 10-00 5-8 6-9 9-1 9-2 1-31 8-40 Average (nemb Average (witho Average (combi	Range   Average   Protein of serum   Serum protein of lymph protein of serum protein of lymph protein of serum protein	in lein
_	The state of the s	

Proteins in Lymph and Serum of Edematous Dogs

		1115 111 Lyma		VI.		
Dog No.	Anesthetic	Predominating	n and Seru	n of Edem	iatous Doe	2
9-92 6 5-8 8-40 9-1 2-3 5 1-31 8-38	B B L L L L L L V Pm.* M	Massage Massage Walking Walking Walking Walking Walking Valking Jassage Jassag	Ranse  fer cent 0.06-0.07 0.15-0.69 0.05-0.25 0.11-0.14 0.34-0.60 0.12-0.25 18-0.24 01-0.08 29-0.53 13-0.60 0	of lymph  Average  per cent 0.07 0.39 0.11 0.13 0.46 0.17 0.22 0.04 0.37 .36	Protein of Serum  Protein of Serum  2.10 2.60 2.49 2.88 3.32 3.38 3.24 3.20 3.00 3.00	

than those in the lymph from normal dogs. A marked reduction in the proteins of serum has also occurred but inspection of the serum protein: lymph protein ratios indicates that the fall in lymph protein has occurred at a more rapid rate than the decline in serum protein. The ratio of average serum protein to average lymph protein in the normal dogs is 3.3; the same ratio in the edematous dogs is 12.7.

### The Protein Content of Lymph and Edema Fluid

In comparing the composition of lymph with that of edema fluid it is important to bear in mind that the larger lymphatic trunks do not drain subcutaneous tissue exclusively. In part tributaries are received from joints, muscles, fascial planes, tendon sheaths, skin, and possibly bone. Among the portions of subcutaneous tissue drained by one vessel all degrees of edema may be present. Lymph collected from such a trunk cannot come exclusively from edematous subcutaneous tissue and it is unlikely that the various tributaries contribute equally to the total volume. Following cannulization the flow of lymph is first rapid and later relatively slow and the rapid flow is associated with a decrease in swelling in the edematous region. fact suggests that the proportionate contributions into the main lymph channel are changing constantly during any period of collection. Finally, it is not unlikely that the continuous exercise necessary for maintaining a flow of lymph may of itself be the cause of variations in capillary permeability and so of fluctuations in composition of the lymph collected. In general the first specimens of lymph will contain a large volume of edema fluid and a small volume of fresh filtrate from the capillaries. Later specimens may be composed chiefly of newly formed filtrate.

In Table VII have been entered the protein concentrations of edema fluid and of the first lymph collected following cannulization. In six instances in which direct comparison is possible (Dogs 5, 8-40, 2-3, 5-8, 1-31) the difference in protein concentration between the two fluids was from 0.02 to 0.09 gm. per cent and averaged 0.04 gm. per cent. The differences are small, as would be expected, although in

<sup>&</sup>lt;sup>6</sup> Specimens from Dogs 8-06 and 8-38 are not compared as they were secured after death. Specimens from Dog 9-1 are also excluded as the edema fluid contained blood.

most instances they are outside the range of analytical error. interesting, then, to note that in four instances of the six the first lymph contained less protein than the corresponding edema fluid. The fact suggests that in some instances the protein in edema fluid may be increased above that in fresh filtrate from the capillaries by further diffusion of protein molecules from the blood during the period of dormancy in the tissues. The fluctuations in lymph protein during periods of continuous collection are presented graphically in Chart 1.

Comparative Prolein Contents of Edema Fluid and the Lymph Collected

* *****	citis of Ria.
Immen	liately after Cannulization  Lymph Collected
	ridlely after Country and the Tour
Hind legs	Cannulization Lymph College
Dog Tegs	Julion Suitected
No. Right	E.
Ed. Left	Fore legs
Hind   Limet   Fd.	Right
fluid Lymph 1	Edema   Ascitic
per fluid Lymph 1	Edema Lymph Edema Lymph finia Lymph
cent per per	fluid Lymph Edema Lymph fluid Nature of edema
m 1 1 (772 1 1 fam 1	
8-40 0 0 0 18 cent cent	
	TOTAL COPY   FET   A. T
24161 + 1 10 2-1 1	cent cent per
0 10 1/ 10 20 1 22 10 111	cent
000111001010.14101	
0.021 -010.531 10.001	0.31 0.02 Nutritional
1 1 1 10301	0.31 0.02 Nutritional
01 1 1 1	10 201 10.43 10 to 1 1 4 4 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
5-8 0.09 0.06	0.07 0.13 Nutritional
9-1 0 860 0.06 0.08	0.07 0.32 Nutritional
10.86*10 = 10.081	10 321 10.00 10 a. 1 "Wiritia"
2-3   1   0.00   0.05   1	0.15 0.01 Plasmaphes
1-31 10 0 1 10 10 1	0.15 0.01 Plasmapheresis
0.16	0.14 Plasmapheresis Plasmaph
* These	Plasmapheresis Nutrition
edema fluid	
nulus contained	0.01 Nutritional
* These edema fluids contained blook No dog in the group included in the	d. See text.
og in the	d. See text. Plasmapheresis
or lymn, Suc group incl.	
" " uph thom " P meluded in a	,

No dog in the group included in the chart produced either edema fluid or lymph the protein content of which exceeded 0.3 gm. per cent. The recorded fluctuations are, therefore, necessarily small. They are considerably less than those encountered in normal dogs. In four instances the lymph protein exhibited a progressive rise as collection was continued. In three of these (Dogs 5, 1-31, 8-10) the rise was slight; in one instance (Dog 5-8) it was strikingly rapid. With Dog 2-3 a different response occurred. Lymph from a hind leg declined progressively in protein content and lymph from a fore leg first increased and then decreased in concentration of this substance. Comparison of these concentrations with those present in edema fluids from corresponding limbs shows that in five instances out of six the edema fluid protein lay within the range of variation of the lymph protein. In the sixth instance (Dog 8-40) lymph protein was constantly higher than edema fluid protein.

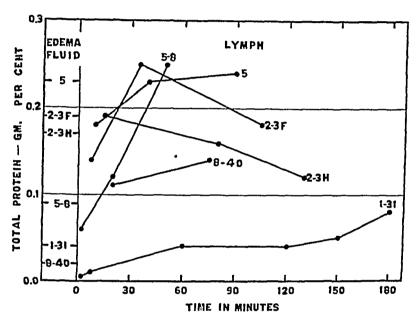


CHART 1. Protein concentration of edema fluid in relation to the fluctuating concentrations of protein in lymph during a period of continuous collection. The numbers identify particular animals, the letters F and H refer to fore leg and hind leg, respectively.

#### The Occurrence of Erythrocytes in Lymph

Systematic microscopic studies of the cellular elements in lymph have not formed a part of this investigation. Such studies have been made by Haynes and Field (11). The gross appearance of all specimens has, however, been noted as a routine; that is, they have been recorded as clear, or slightly, or moderately discolored with red blood cells. At first the possibility was entertained that these cells might be finding their way into the lymphatics through absorption from the tissues in the region of the operative incision. We have failed, however, to secure evidence in support of this possibility. In one experi-

ment the lymphatics of both fore legs were cannulated. On the right side no attempt was made to control hemorrhage during the operative manipulations and blood was purposely allowed to collect about the edges of the field. On the left side hemorrhage was controlled both by hemostats and with adrenalin. Lymph obtained from both limbs was entirely clear. In other experiments the operative field has been sopped with India ink or the ink has been injected subcutaneously in the same region before making the initial incision. In no case has the ink appeared in the lymphatics from which collection was being made. Although the majority of lymph specimens do not contain blood cells, their presence has been observed in samples collected both from normal and edematous dogs, both with and without general anesthesia, and when lymph flow was being stimulated either by massage, passive motion, or normal activity. We have come, therefore, to regard the finding as a normal physiologic phenomenon which, as such, may assist toward an understanding of the blood capillary - tissue space → lymphatic capillary circulation.

# Summary of Experimental Observations

Lymph flow in normal dogs ceases entirely during periods of complete physical inactivity. During these periods capillary filtrate ac-Cumulates in the interstitial spaces and can enter the lymph channels at once when activity stimulates the pumping action of the lymphatic valves. The initial flow is, therefore, rapid but the rate declines quickly as the interstitial reservoir is emptied and finally becomes constant at a rate which corresponds to that at which new lymph is being produced. With the edematous dog the situation is similar but because the interstitial spaces contain more fluid (edema) the initial rapid flow can be maintained for a longer time than in the normal animal. Within 10 or 15 minutes, however, the rate of flow decreases and continued activity is accompanied by progressive and finally by complete loss of edema. The carrying capacity of the lymph vessels at all times greatly exceeds the rate at which new lymph can be formed. The data suggest that the rate of lymph formation, as estimated from the minimum rates of lymph flow, may increase slightly when edema is present. The increase, however, is surprisingly small and not beyond the limits of variations encountered in normal animals.

Lymph from the normal dog always contains an appreciable quantity of protein. Lymph from the edematous dog contains much less protein. The lymph protein deficit of edematous dogs is greater than can be accounted for on the basis of a proportionate loss corresponding to the serum protein deficit. The concentration of protein in lymph from edematous dogs is of the same order of magnitude as that of edema fluids although the two fluids are not identical in composition. Minor fluctuations in the protein of lymph occur while the collections are being made. The fluctuations may depend on varying proportionate rates of flow from different regions which send tributaries to the main lymph channels or they may result from variations in capillary permeability incident to the continuous exercise necessary for maintaining lymph flow.

Lymph from the lower leg of normal and edematous dogs sometimes contains red blood cells and sometimes it does not. Both increases and decreases in the number of erythrocytes may follow in succession as the conditions of collection are altered.

#### DISCUSSION

The theory of Starling (1) postulates that the exchange of fluid across the capillary wall is controlled by a balance between capillary blood pressure, which acts to force fluid from the capillary into the tissues, and colloid osmotic pressure of the blood plasma, which tends to draw fluid from the tissues into the capillary. The balance is adjusted so that mechanical pressure is greater than osmotic pressure at the arterial end of the capillary and less than osmotic pressure at the venous The gradient in pressure through the capillary leads to filtration of fluid at the arterial end and reabsorption of the fluid at the venous end. When filtration and absorption are equal, as is assumed in health, fluid, which is in effect an ultrafiltrate of plasma, circulates constantly through the tissues and so favors the free exchange of substance. The theory was elaborated in the experiments of Schade (4) on model capillaries made of collodion with which he demonstrated both filtration and reabsorption when the collodion tubes were perfused with serum under controlled pressure. In 1930 Landis (12) published the results of measurements on vessels at the base of the human finger nail. In a series of manometric determinations by a microinjection technique he showed an average fall in pressure from 32 mm. of mercury at the arterial end of capillaries to 12 mm. at the venous end. As the colloid osmotic pressure of the serum of human beings varies between 23 and 28 mm. of mercury, the conditions anticipated by Starling were realized in the measurements. From the quantitative standpoint information is still lacking regarding the volume of fluid which circulates through the tissue spaces as a

result of the pressure gradient in the capillaries. It has seemed to us, however, that this volume must be small in comparison with the movement of fluid which results when the flow of lymph is stimulated by physical exercise or massage.

In a previous communication (10) it was stressed that the movement of fluid across the capillary wall is controlled by forces on the outside as well as on the inside of the wall. The effective colloid osmotic pressure is the difference between the osmotic pressure of serum (S) and the osmotic pressure of the interstitial fluid (I), whereas the effective mechanical pressure is the difference between capillary blood pressure (C) and back pressure from the tissues (T). Under stationary conditions, that is at times when edema is neither forming nor receding, the forces are in a state of equilibrium expressed by the equation:

Recently Landis and Gibbon (13) have called attention to the necessity of considering tissue pressure in order to explain reasonably the diminishing rate of increase in arm volume following a given elevation in venous pressure. In view of the findings in this investigation it becomes obvious that the state of equilibrium expressed by the above equation can be attained only during periods of complete rest; that is, at times when there is no perceptible flow of fluid through the lymph channels. During rest fluid accumulates in the subcutaneous tissue until the attending rise in pressure leads to a balance between the processes of filtration and reabsorption. That some extracapillary fluid does accumulate during rest even in normal animals under normal conditions of venous pressure may be assumed from the evanescent rapid flow of lymph which accompanies the onset of activity.\* The

Knowledge of the factors which govern tissue pressure is very inadequate. Recently Holland and Meyer (Holland, G., and Meyer, F., Arch. exp. Path. u Pharmakol., 1932, 168, 603) have presented measurements upon which they base the conclusion that marked edema is not attended by a disturbance of tissue pressure. In the discussion which follows we are concerned with immediate variations in pressure which may be assumed to follow changes in lymphatic activity and not with the question of remote changes which might be conceived to result from the operation of forces responsible for edema.

From observations through a transparent chamber in the rabbit's ear, Clark and Clark (Clark, E. R., and Clark, E. L., Am. J. Anat., 1933, 52, 273) have concluded that under usual conditions no free fluid is present in the tissue spaces. If

efficiency of the lymphatic pump must be considerable for, as we have seen, markedly edematous areas can be reduced to a non-edematous state within several hours. The removal of tissue fluid must lead to an immediate fall in pressure in the tissue spaces and disturb the relation between capillary filtration and reabsorption in such a way as to increase the quantity of fluid actually leaving the capillaries. That is to say, the drop in pressure will lead to an increase in the area of capillary wall functioning for filtration and to a decrease in the surface available for reabsorption.

Under these circumstances it is pertinent to inquire whether during physical activity the process of capillary reabsorption may not be completely in abeyance and the whole of the capillary wall function for filtration. That such may be the case is suggested by the failure to observe any considerable increase in the rate of lymph formation in animals exhibiting serum protein deficits. The Starling theory demands that a declining osmotic pressure of the plasma be accompanied by a steadily increasing area of capillary wall available for filtration. For this reason alone an accelerated lymph formation should be expected in the presence of hypoproteinemia. The failure to measure such an increase may, therefore, be taken to suggest that the whole of the permeable surface of the capillary already functions for filtration during periods of physical activity in the normal animal. Furthermore, regardless of explanation, the finding also suggests that failure of the mechanism for reabsorption plays a greater rôle in the causation of edema than increase in the rate of filtration.

The equation given on page 79 also expresses the fact that the effective colloid osmotic pressure of the serum is represented by the difference between the total osmotic pressure of serum and the osmotic pressure of the fluid on the outside of the wall of the capillary. If the wall of the capillary were almost impermeable to colloids, as was believed by Starling (1), Schade (4), Krogh (3), and others, the fluid outside the wall would contain so little protein that its effective osmotic pressure would be negligible. At the present time, however, the extensive evidence assembled by Drinker and Field (8) is too convincing to allow continuance

their observations should be shown to hold for the subcutaneous tissues in general, it would become necessary to think that the fluid which accumulates outside the capillaries during rest is located within the lymphatic capillaries and not strictly speaking in the tissue spaces.

A. A. WEECH, E. GOETTSCH, AND E. B. REEVES of belief in the impermeability of all capillaries at all times to protein. Krogh, Landis, and Turner (14) have pointed out that the summation of the processes of filtration and reabsorption may mean that the filtering surface of the capillary is bathed in a fluid of low protein content whereas at the absorbing surface the concentration may be raised considerably. They suggest that lymph may represent the fluid remaining after the process of reabsorption is complete and are unwilling to look upon it as representative in composition of average tissue fluid. According to them the experience of several authors with mechanical filtration edemas indicates that extravascular fluid often contains less than 0.1 per cent protein and they believe that this figure represents more closely the composition of capillary. We have seen, however, that lymph often contains more than 1 per cent protein. Aside from the fact that it is difficult to believe that bordering layers of fluid in the tissue spaces can differ so markedly in composition, it may be noted that reasons have already been given for supposing the process of capillary reabsorption to be in complete abeyance during periods of active functioning of the Sorption to be in complete abeyance during periods of active functioning of the land of th composition of lymph became constant between levels of 0.5 to 1.52 per cent of protein. Our experience has been similar. It might be reasoned, then, that these figures provide a closer approximation of the composition of capillary filtrate in normal dogs at least. In a previous communication, however, the present authors (15) have given another reason for being unwilling to accept as proven the identity of tissue fluid and lymph. It was possible to think that only occasional capillary loops permitted the passage of protein, that this protein did not diffuse generally into surrounding structural units, but rather that it passed at once into the nearest Into surrounging structural units, but rather that it passed at once into the nearest lymphatic radicle. As there was reason to believe that the volume of filtrate would be greater from those capillaries which allowed protein to pass than from those which did not, and, as the lymph collected from a large trunk represented a mix-Which old not, and, as the lymph confected from a large trunk represented a mixture of the streams coming from many querent radicies, it appeared evident that this lymph might be representative neither of average tissue fluid nor of average lymph in the many different radicles. As yet no completely satisfactory method of testing the validity of this theory has been devised. Nevertheless, the observations on the occurrence of erythrocytes in lymph strongly support the belief that cocasional capillaries may exhibit an unusual degree of permeability. In fact, Occasional capillaries may exhibit an unusual degree of permeability. In fact, Orinker and Field (8) have stated that "the red-cell content of lymph is probably of the processing and as making some have some the changement in the such gross." If such gross rupture is possible, and, as we have seen, the phenomenon is not infrequent, it is not unreasonable to think that dilated pores of a size sufficient to permit the passage of protein may be even more frequent. It is obvious that the principle insage of protein may be even more frequent. It is obvious that the principle in-Permeable to protein. It does, however, imply that permeability and rate of filpermeable to protein. It does, nowever, imply that permeability and rate of intration may vary between different capitaines in such a way that tymph collected from a larger trunk fails to be representative in composition of either capillary. filtrate or tissue fluid. trate or tissue name.

The finding in this investigation that the protein deficit of lymph from edema-

ous animals is too great to be accounted for on the basis of hypoproteinemia lone, has suggested two possible explanations: (1) Either as a result of hypoprosinemia or because of distention of the tissue spaces with edema, the permeability f the capillaries for protein may be lessened. (2) The fall in plasma osmotic ressure may so increase the filtering area of capillary wall and decrease the resorpive area that the reduction in lymph protein is due not only to the decrease in lasma protein but also to a further dilution with water which in the normal nimal would be reabsorbed into the capillaries. An assumption of diminished ermeability of the capillaries has to us been the more acceptable explanation for he reason that an increase in the filtering area of capillary wall would be expected o increase the volume of capillary filtrate, and we have seen that the rate of lymph ormation in edematous animals does not give evidence of such an increase.

Comparative analyses of lymph and edema fluid in this investigaion have revealed an approximate identity in the composition of the The finding lends some support to the belief that lymph wo fluids. nay be regarded as representative in composition of average tissue uid. Under the circumstances it is perhaps most wise not to stress light differences in composition which were, however, outside of the ange of analytical accuracy. Nevertheless, to our minds, the differnces have been of such a nature as to emphasize the manner in which ransient changes in capillary permeability (as may occur during exerise) or variations in the quantity of lymph coming from different tructural units in the tissues (as certainly occurs when the supply of ree edema fluid has been exhausted) are reflected in immediate fluctuaions in the quantity of protein in lymph. Under such circumstances he data do not as yet warrant the conclusion that lymph and tissue luid from normal animals will agree as closely in composition as lymph nd edema fluid from animals with serum protein deficits.

#### SUMMARY

1. The experimental observations have been summarized at the end of an earlier section. The more important facts only will be recapituated here.

The capacity of the lymphatics for removing fluid from the tissues creatly exceeds the rate at which freshly formed tissue fluid can be nade available for removal. Edematous regions can be rendered non-edematous by the application of measures, such as massage, pasive motion, or normal exercise, which activate the lymphatics.

During continuous activity the rate of lymph flow is first variable and later relatively constant. Constant rates of flow must correspond 83 to the production of fresh lymph. A study of the constant rates indicates that lymph formation in the edematous animal is certainly only slightly greater, and possibly not greater at all, than under conditions of normality.

When the protein of plasma decreases, the protein of lymph is also lowered. The loss of protein from lymph takes place at a faster rate than from plasma, so that the ratio of serum protein to lymph protein is greater in the edematous than in the normal animal.

In edematous animals the concentration of protein in lymph is of the same order of magnitude as the concentration in edema fluids. The two fluids are not, however, identical in composition. fluctuations in the protein content of lymph always occur during a period of continuous collection.

2. The factors involved in the circulation and accumulation of tissue fluid are discussed. Reasons are given for offering the following sug-

Significant differences in tissue pressure or tension exist between the states resulting from quiescence and activation of the lymphatics. The differences give rise to variations in the relative areas of capillary wall, functioning for filtration and reabsorption. When the lymphatics are activated it is possible that capillary reabsorption may be

A decline in the proteins of plasma may be associated with a diminished permeability of the capillaries. Such a lowering of capillary permeability would account for two features, both of which have been demonstrated: (1) failure to observe an appreciably increased rate of lymph formation in the edematous animal, and (2) the extremelylow concentration of protein in lymph from edematous animals.

Although the difference between the protein concentrations of edema fluid and lymph from the same region is small, the conclusion is not yet justified that a similarly small difference exists between normal

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# CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

VIII. THE INFLUENCE OF THE ACETYL GROUP ON THE SPECIFICITY OF HEXOSIDE-PROTEIN ANTIGENS

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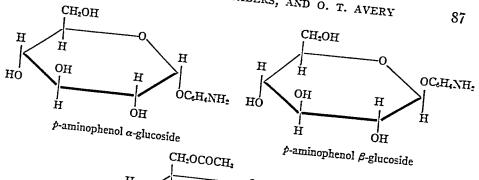
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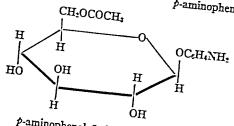
That the acetyl group (CH<sub>3</sub>CO-) can exert a specific influence in determining the serological characteristics of a bacterial carbohydrate has recently been shown in this laboratory (1). The capsular polysaccharide of Pneumococcus Type I occurs in the intact cell as an acetyl derivative of a complex nitrogenous carbohydrate. of the acetyl groups with the polysaccharide itself is of a highly labile nature. When these groups are removed by mild alkaline hydrolysis, the resultant deacetylated carbohydrate is found to be deprived of certain immunological properties possessed only by the parent substance. Since the only demonstrable chemical change that occurs during alkaline hydrolysis is the removal of acetyl groups, the additional serological characteristics exhibited by the fully acetylated carbohydrate can be attributed only to the presence of the acetyl radical in the intact bacterial polysaccharide.

In the attempt to understand the immunological significance of the acetyl group in the more complex bacterial polysaccharides, a study has been made of the serological specificity of two related antigens each containing a simple carbohydrate radical in the acetylated and the unacetylated form. For this purpose, therefore, the p-aminophenol  $\beta$ -glucoside of glucose and its monoacetyl derivative have been synthesized. These two glucosides have been combined with the globulin of horse serum by means of the diazo reaction, and the resultant

"synthetic" carbohydrate azoproteins have been employed as antigens in the production of immune rabbit sera. The carbohydrate radicals of these two antigens are identical in their stereochemical relationships, yet they differ in that one of the derivatives contains an acetyl group. From the mode of synthesis it is probable that the acetyl radical is bound on the sixth carbon atom of the glucoside, although its exact allocation in the molecule is, for the purpose of this study, irrelevant.

It has previously been shown that conjugated carbohydrate-protein antigens containing either the azophenol  $\alpha$ - or  $\beta$ -glucoside give risc in each instance to antibodies which are predominantly specific, yet capable of a secondary reaction with test antigens containing the heterologous hexoside (2). By means of the inhibition test it has been shown that the primary immune reaction is quite specific, since the union between homologous antigen and antibody is inhibited only by homologous glucoside, whereas the cross-reaction with the heterologous antigen is inhibited by both the homologous and the heterologous glucosides. Thus a difference in the stereochemical configuration of the carbon atom bearing the aglucon suffices to determine the immunological specificity of the  $\alpha$  and  $\beta$  derivatives of glucose irrespective of the protein with which they are combined. The identity in structure of the remaining five carbon atoms of both these glucosides may account for the cross-reactions between the antisera and the heterologous hexoside-protein antigen. If the structural identity of the terminal portion of the hexoside molecule be altered through the introduction of a chemical grouping such as the acetyl radical, then one might anticipate that the immunological specificity of the altered glucoside might well be different from that of the same glucoside in its unacetylated form. That this phenomenon actually occurs will be seen from the following experimental data. parison the immunological properties of an antigen containing azophenol \alpha-glucoside are included. The structural relationships of the three hexosides,  $\alpha$  and  $\beta$  p-aminophenol glucosides and the acetyl derivative of the latter, are illustrated by the following graphic formulae:





p-aminophenol  $\beta$ -glucoside monoacetate

## EXPERIMENTAL

1. p-Nitrophenol β-Glucoside Monoacetate.—10 gm. of p-nitrophenol β-glucoside I. CHEMICAL were dissolved in 30 cc. of anhydrous pyridine. The solution was cooled to  $0^{\circ}$ and 2.61 cc. (1.1 mols) of acetyl chloride were slowly added. The mixture was kept cold for several hours and then allowed to stand at room temperature for 3 or 4 days. The pyridine was removed by distillation in vacuo and the residual syrup evaporated several times with absolute ethyl alcohol. The mixture was allowed to stand for 24 hours at ice box temperature to facilitate crystallization. Crystals of p-nitrophenol  $\beta$ -glucoside monoacetate were filtered from the mother liquors and washed with cold ethyl alcohol. 3.4 gm. were recovered. The compound was recrystallized several times from ethyl alcohol. The substance melted Analysis: 4.863 mg. substance gave 8.410 mg. CO2 and 2.165 mg. H2O.

C<sub>14</sub>H<sub>17</sub>O<sub>9</sub>N Calculated: C 49.00 per cent; H 5.00 per cent.

8.236 mg. substance when analyzed by Pregl's (3) method for the determination of acetyl groups, utilized 1.65 cc. N/70 NaOH.

CH<sub>3</sub>CO- Calculated: 12.53 per cent. Found: 12.32 per cent.

$$[\alpha]_{p}^{=6} = \frac{-2.33^{\circ} \times 100}{2 \times 10 \times 0.1102} = -105.7^{\circ} \text{ (in methyl alcohol)}$$
Thereof  $\beta$ -Glucoside Man

2. p-Aminophenol β-Glucoside Monoacetate.—2.0 gm. of p-nitrophenol β-gluco-

side monoacetate were dissolved in 100 cc. of warm methyl alcohol. The substance was reduced catalytically with 50 mg. of platinum oxide and hydrogen according to the method of Vorhees and Adams (4). From the alcoholic solution 1.2 gm. of p-aminophenol  $\beta$ -glucoside monoacetate were isolated. This compound crystallized as a snow-white product difficultly soluble in ethyl alcohol, more soluble in methyl alcohol, and much more easily soluble in water. It melted at 177–179°C. (uncorrected) and had a specific optical rotation of

$$[\alpha]_{D}^{21^{\circ}} = \frac{-1.69^{\circ} \times 100}{2 \times 10 \times 0.1204} = -70.2^{\circ}$$
 (in methyl alcohol)

Analysis: 8.806 mg. substance when analyzed for acetyl groups, utilized 1.94 cc. N/70 NaOH,

CH<sub>3</sub>CO- Calculated: 13.75 per cent.

Found: 13.55 per cent.

32.3 mg. sample when analyzed for nitrogen by the micro Kjeldahl method utilized 6.87 cc. N/70 HCl.

N Calculated: 4.47 per cent.

Found: 4.26 per cent.

The monoacetate of p-aminophenol  $\beta$ -glucoside is relatively stable to alkaline hydrolysis. Aqueous solutions must be warmed (40-50°) with n/10 alkali before the acetyl group begins to hydrolyze.

- 3. p-Aminophenol  $\alpha$  and  $\beta$ -Glucosides.—These compounds were prepared by methods previously described (2, 5).
- 4. Preparation of Protein Azophenol  $\alpha$ -Glucoside,  $\beta$ -Glucoside, and  $\beta$ -Glucoside Monoacetate Antigens.—
- (a) Immunizing Antigens.—The amino glucosides were combined with the globulin of normal horse serum in the manner previously described (2). The solutions of sugar-azo protein were sterilized by filtration through a Berkefeld candle and were used for immunization of rabbits.
- (b) Test Antigens.—In order to avoid the possibility of antiprotein precipitins masking the specificity of the anticarbohydrate reactions, the test antigens used in the precipitin reactions were prepared by combining the respective glucosides to the proteins of chicken serum.

#### II. IMMUNOLOGICAL

#### Methods

Rabbits were immunized by the intravenous injection of solutions of the conjugated hexoside-protein antigens. The rabbits of one series received the antigen composed of horse serum globulin coupled to diazophenol  $\alpha$ -glucoside, those of another series were treated with the same protein combined with diazophenol  $\beta$ -glucoside, while those of a third series received the protein in combination with the acetyl derivative of the  $\beta$ -glucoside. The rabbits of all three series were in-

jected intravenously with 1 cc. of the respective antigen, containing 10 mg. pro-89 tein per cc., daily for six doses. The course of injections was repeated after a rest period of 1 week. 8 days following the last injection the rabbits were bled and the sera tested for precipitins against the homologous and heterologous test antigens. The technique of the precipitin and inhibition tests is the same as that

## Carbohydrate Antibodies

1. Homologous Precipitin Reactions.—The sera of rabbits immunized with the carbohydrate-protein antigens were first tested for the presence of homologous precipitins against test antigens containing the homologous glucosides combined with the protein of chicken serum.

The precipitin reactions between the homologous antisera and the corresponding test antigens are summarized in Table I. The results

Homologous Precipitins in Sera of Rabbits Immunized with  $\alpha$ -Gluco-Globulin, β-Gluco-Globulin, and Acetyl β-Gluco-Globulin

Antiserum	modulin, and Acetyl β-Gluco-Globulin α-Gluco-Globulin,						
1. α-Gluco-globulin	Test antigen	Dilution of test antigen					
<ol> <li>β-Gluco-globulin</li> <li>Acetyl β-gluco-globulin</li> </ol>	α-Gluco-chicken serum	1:5,000 1:10,000 1:20,000					
	Acetyl &-gluco-chicken	+++±++± ++± +++++++++++++++++++++++++++					
++++= Complete prec	ipitation with disk-like	+++ ++					

<sup>++++ =</sup> Complete precipitation with disk-like precipitate.

of the homologous precipitin tests illustrate the property of the immune serum to react with an antigen containing the homologous glucoside, irrespective of the protein with which it is combined. It is further evident that the diazophenol glucosides when combined with protein function as excellent antigens.

2. Heterologous Precipitin Reactions.—In order to ascertain whether the antisera interact with the different carbohydrate antigens, the respective sera were tested against each of the heterologous test antigens. The results are summarized in Table II. From the results given in Table II, it may be seen that the immune serum obtained by immunization with an antigen containing the  $\alpha$ -glucoside gives rise to

antibodies which cross-react with a test antigen containing the unacetylated  $\beta$ -glucoside, but not with one containing this glucoside in the acetylated form. On the other hand, an antiserum produced by immunization with  $\beta$ -gluco-globulin reacts not only with the homologous test antigen, but with  $\alpha$ -gluco- and acetyl  $\beta$ -gluco-antigens as well. If one of the hydroxyl groups of the immunizing antigen containing the  $\beta$ -glucoside is covered with an acetyl group, then the homologous antiserum, although it still reacts with a  $\beta$ -gluco-antigen, fails to react with the  $\alpha$  homologue. The chemical basis underlying

TABLE II

Heterologous Precipitins in Sera of Rabbits Immunized with α-Gluco-Globulin,
β-Gluco-Globulin, and Acetyl β-Gluco-Globulin

Antiserum	Test antigen	Dilution of test antigen			
	Test unitgen	1:5,000	1:10,000	1:20,000	
1. α-Gluco-globulin	α-Gluco-chicken serum β-Gluco-chicken serum Acetyl β-gluco-chicken serum	+++± +++ 0	++± +± 0	+± + 0	
2. β-Gluco-globulin	α-Gluco-chicken serum β-Gluco-chicken serum Acetyl β-gluco-chicken serum	++ +++± ++±	+± +++ ++	+++++	
3. Acetyl β-gluco-globulin	α-Gluco-chicken serum β-Gluco-chicken serum Acetyl β-gluco-chicken serum	0 +± ++±	0 + ++	0 + ++	

<sup>++++ =</sup> Complete precipitation with disk-like precipitate.

the differences in the immunological properties of these glucosides will be discussed later.

3. Specific Inhibition Tests.—The selective specificity of each of the gluco-protein antigens is clearly demonstrated by the results of the inhibition tests which are given in Tables III, IV, and V. Analysis of the data presented in Tables III, IV, and V shows in each instance that upon addition of the corresponding glucoside to its homologous immune serum the specific precipitins are completely bound and are no longer capable of reacting with either the homologous or heterolo-

<sup>0 =</sup> No precipitation.

Specific Inhibition of Precipitins in Acetyl \(\beta\)-Gluco-Globulin Antiserum by Homol-

-		7	ogous	and Het	erologous	Gluc	±1001llin Osidoc*	Anliser	um by Homol-
Acet	Acetyl-p-gluco- globulin antiserum		p-aminophenol glucosides  1/10			T	7		
			β Acetyl β		Salt solu- tion to volume	precipita-	Acetyl	t antigen :5,000)*	Part
Test No	ο. α.	α.	cc.	α.		ê	β-gluco- chicken serum	β-Gluco- chicken serum	Result
2	0.2	_	-	_	0.3	ا القار	cc.	cc.	
3 4 5	0.2		_	0.3	0.3	- 1	0.5	 0.5	++++
6	0.2	-	0.3	0.3	- 2	- 1	0.5	0.5	0 0
8	0.2	0.3	-	_	I       Incubated		-	0.5	+++
*α-Gl act in ac	uco-chicl etyl β-gl	ken serui	m antige	n is not i	included i			0.5	+++± +±

<sup>\*</sup>  $\alpha$ -Gluco-chicken serum antigen is not included in this table since it fails to react in acetyl  $\beta$ -gluco-globulin antiserum (cf. Table II).

Specific Inhibition of Precipitins in  $\alpha$ -Gluco-Globulin Antiserum by Homologous

		ana	Heleral	C-Giuco-Glo	bulin $An$	disare			
a-Gluco-globulin antiserum	n/10 glucosides			1					
Test No. 4.	α	β	Acetyl B	Salt solu- tion to volume	didicki	(1:5,000)*	Per Result		
1 0.2	<i>τ</i> ε.	cc.	cc.	-α.   i		chicken serum	. 1		
$\begin{bmatrix} 0.2 \\ 0.2 \\ 0.2 \\ 0.2 \end{bmatrix}$	$\begin{bmatrix} - \\ 0.3 \\ 0.3 \end{bmatrix}$	-	-	2.0 8. at 37°C	_	0.5	  ++++		
$\begin{array}{c c} 6 & 0.2 \\ 7 & 0.2 \\ 0.2 & 0.2 \end{array}$	-	0.3	-		0.5	0.5	++± 0 0		
* Acetyl B. gluco		=	0.3	 Incubated	-	0.5	+++		
*Acetyl \(\beta\)-gluco to react in \(\alpha\)-gluco-	chicker globulii	serum.	antigen i	S not incl.		0.5	++ ++		

<sup>•</sup> Acetyl  $\beta$ -gluco-chicken serum antigen is not included in this table since it fails to react in a-gluco-globulin antiserum (cf. Table II).

gous test antigens. The specificity of the inhibition reaction is demonstrated by the fact that a heterologous glucoside does not inhibit the homologous precipitin reaction. The acetylated  $\beta$ -glucoside antigen induces an immune response which is specifically distinct from that induced by an antigen containing this glucoside in its unacetylated form, for it is seen that the unacetylated  $\beta$ -glucoside fails to inhibit the reaction between the acetyl  $\beta$ -glucoside antigen and its homologous antibody (Test 5, Table III). This fact indicates clearly that the

TABLE V

Specific Inhibition of Precipitins in β-Gluco-Globulin Antiscrum by Homologous and

Heterologous Glucosides

β-Gluco-globulin antiserum		p-Aminophenol glucosides			Salt		Test	5,000)		
		α	β	Acetyl \$	or o		β-Gluco- chicken serum	β-Acetyl- gluco- chicken serum α-Gluco- chicken serum		Result
Test No.	cc.	cc.	cc.	cc.	cc.	prec	cc.	cc.	cc.	
1	0.2				0.3	-no	0.5		_	++++
2	0.2	<b> </b>		-	0.3	ن	_	0.5		+++
3	0.2	-	l —		0.3	37°			0.5	++
4	0.2		0.3			at .	0.5	_	-	0
5	0.2		0.3			hrs.		0.5		0
6	0.2		0.3					-	0.5	0
7	0.2	0.3		-	- :	d 2	0.5	}		+++±
8	0.2	0.3		-		ate		0.5		++
9	0.2	0.3				멸	- 1	- 1	0.5	0
10	0.2			0.3	}	Incubated	0.5	[		++++
11	0.2			0.3	[		-	0.5		0
12	0.2	-	-	0.3					0.5	+± ———

acetyl group confers a distinct and additional specificity upon a simple carbohydrate the stereochemical structure of which remains unaltered. From the results in Table IV, it is seen that the acetyl  $\beta$ -glucoside does not inhibit the reaction of the unacetylated  $\beta$ -gluco-test antigen in  $\alpha$ -gluco-globulin antiserum (Test 8). In Table V it may also be seen that the reaction between  $\beta$ -gluco-globulin antiserum and the homologous antigen, although inhibited by the  $\beta$ -glucoside (Test 4), is not inhibited by the same glucoside when one of the hydroxyl groups has been covered by an acetyl radical (Test 10).

The results of the present study not only confirm the view previously DISCUSSION held that the immunological specificity of carbohydrates is determined by their stereochemical configuration, but they lend support to the further assumption that the introduction of a simple chemical group, such as the acetyl radical, endows a carbohydrate with a new and distinct specificity which is determined by the chemical nature of the group thus introduced. It has been previously pointed out that differences in the specific behavior of  $\alpha$ - and  $\beta$ -glucosides of glucose may be accounted for by differences in the stereochemical configuration of the carbon atom bearing the aglucon, and that the basis for the immunological crossing may lie in the fact that the spatial configuration of the polar groups on the remaining five carbon atoms is identical. This explanation appears to be further supported by the results of the present study. For it can be seen from Table II that when  $\beta$ -glucotest antigen, which normally reacts in  $\alpha$ -gluco-globulin antiserum, is so altered that one of the polar groups (OH) of the five terminal carbon atoms of the carbohydrate radical is replaced by acetyl (CH<sub>3</sub>CO), the resulting antigen fails to react in  $\alpha$ -gluco-globulin antiserum.

Furthermore,  $\alpha$ -gluco-test antigen, which normally reacts with  $\beta$ gluco-globulin antiserum, fails to react in the immune serum produced by immunization with acetyl  $\beta$ -gluco-globulin. Similarly, the acetylated  $\beta$ -glucoside, due to the alteration in chemical constitution, fails to bind the antibody in  $\alpha$ -gluco-globulin antiserum, and as a result permits both the  $\alpha$ - and  $\beta$ -gluco-test antigens to react in their normal course. The  $\beta$ -glucoside likewise fails to inhibit the reaction between the acetyl  $\beta$ -gluco-test antigen, and homologous immune serum. This difference in the serological specificity of the antibodies induced by  $\beta$ -gluco-antigens in the acetylated and unacetylated form can be attributed only to the known differences in the chemical structure of these two glucosides. The latter differ from one another in that the acetyl  $\beta$ -glucoside is a derivative in which one of the polar groups (OH) of the carbohydrate has been altered by the introduction of an acetyl

A critical analysis of the results of these serological tests again emphasized the fact that the presence of an acetyl group in a carbohydrate exerts a determining influence on the specificity of an antigen of which it forms a part. In conclusion it may be pointed out that the differences in serological specificity exhibited by the acetylated and deacetylated polysaccharides of Type I Pneumococcus are accurately paralleled by the purely synthetic system described.

#### SUMMARY

The chemical and immunological properties of the acetylated and unacetylated forms of the p-aminophenol  $\beta$ -glucoside of glucose have been described. The serological specificity of these  $\beta$ -glucosides in combination with protein has been correlated with known changes in chemical structure and has been compared with the immunological properties of the  $\alpha$ -glucoside of the same hexose.

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## THE RESPIRATION MECHANISM OF PNEUMOCOCCUS. III\*

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(Received for publication, April 11, 1934)

In two previous communications on the respiratory mechanism of the pneumococcust the following observations were made. A washed suspension of various types and strains of pneumococcus uses up oxygen in the presence of glucose, sodium lactate, ethyl and propyl alcohols, and glycogen. The oxygen thus consumed is quantitatively converted into hydrogen peroxide as shown by analysis after a 10-20 minute reaction period. Later a discrepancy occurs, since the per cent of hydrogen peroxide found is less in proportion to the volume of oxygen consumed. This discrepancy is caused by an intermediary reaction between hydrogen peroxide and pyruvic acid, these being formed simultaneously from glucose or lactic acid. In the case of lactic acid the reaction forms one molecule of acetic acid, one molecule of carbon dioxide, and one molecule of water. Besides these reaction products, we have isolated another acid of unknown constitution. tions involved in the formation of this acid we assume to be responsible for the excess of hydrogen peroxide found during the oxidation of lactic acid. Should lactic acid be quantitatively oxidized to hydrogen peroxide and pyruvic acid, they would react with one another at the time of their formation, and would be absent in the reaction mixture. However, since there is an excess of hydrogen peroxide, it must be assumed that the quantity of pyruvic acid formed is less than that of hydrogen peroxide, and the presence of this excess peroxide must be attributed to another source.

<sup>•</sup> This work was made possible through the financial support of the Rockefeller Foundation.

<sup>&</sup>lt;sup>1</sup> Sevag, M. G., Ann. Chem., Berlin, 1933, 507, 92; Biechem. Z., Berlin, 1933, 267, 211.

Because of the destruction of the enzyme system by the excess hydrogen peroxide, the consumption of oxygen begins to slow down after 20-30 minutes, and the system is paralyzed entirely after 60-80 This destruction is accounted for by the lack of catalase in the cellular substance, or the lack of ability of the organism to complete the reaction and to convert lactic acid and glucose quantitatively into hydrogen peroxide and pyruvic acid. These observations bring up the question: What will be the oxygen-consuming capacity of various organisms if we supply them with protective agents such as catalase and pyruvic acid?2 The addition of 1 cc. of colorless, clear, blood catalase, or 1 cc. m/5 sodium pyruvate to the system increases the oxygen consumption up to 1772 per cent and with pyruvic acid up to 920 per cent within 300 minutes. The rôle of catalase and pyruvic acid as protective agents for enzyme systems is evident. Not only do they enable the organisms to carry on their respiratory functions, but also to maintain their reproductive functions and virulence for a longer period of time. The organism in a reaction system consisting of phosphate buffer and a fermentable substrate, after a 3 hour reaction period failed to grow in a new medium, and failed to kill mice. On the other hand, in the presence of catalase and pyruvic acid the organism maintained its ability to respire, and to grow in a new medium and to kill mice. Furthermore the activity of the organism respiring in a system containing glucose or lactic acid as substrate, and catalase or pyruvic acid as protective agent is many times greater than the activity of those organisms which respire in a similar system free from a fermentable substrate. In the absence of a substrate the organisms are deprived of a source of energy necessary for self-maintenance, whereupon they undergo oxidative autolysis, resulting in a diminution of the number of active reproductive forms. usually associated with a loss of virulence, although the organisms are still viable.

In the presence of protective agents the capacity of various types and strains of pneumococci to consume oxygen is practically of the same magnitude. The slight discrepancies do not offer sufficient basis for the formation of a definite opinion concerning their metabolic

<sup>&</sup>lt;sup>2</sup> Sevag, M. G., Naturwissenschaften, 1933, 21, 466-467.

This similarity is only apparent, and is due to the early destruction of the organism by hydrogen peroxide. Noteworthy-97 differences are first observed when we supply the respiring system of each organism with protective agents. They remove the hydrogen peroxide immediately after its formation, and hence the organisms are able to survive the experiment. By this means sharp differences can be observed (1) between various types, (2) between various strains belonging to the same type, (3) between virulent and avirulent organisms belonging to the same type, and finally (4) between young and old avirulent organisms of the same strain. It has also been shown that a young avirulent organism derived from Type I virulent (75) consumed many times more oxygen than the old avirulent organism derived from Type I virulent (Laux). We were not able to decide whether this difference was due to individual characteristics inherited from the various virulent parent strains, or was true in young and old avirulent mutants derived from a single virulent parent strain. In the present paper we have attempted to do this.

#### EXPERIMENTAL

The consumption of atmospheric oxygen was measured by means of a Barcroft-Warburg apparatus, using Brodie's solution as a manometer liquid. For the respiratory system the bacterial sediment was prepared as follows: To obtain the virulent organisms beef broth was inoculated with the heart's blood of an infected mouse and incubated for 10 hours, after which 0.1-0.5 cc. was introduced into 100 cc. of 0.1 per cent glucose bouillon pH 7.6, and incubated for 10 hours. Such 10 hour old cultures were used in all the experiments. They were centrifugalized as quickly as possible, and the sediment was suspended in 11/15 phosphate buffer pH 8.0, and centrifugalized. The sediment from the second centrifugalization was suspended in the desired volume of the same buffer and used immediately.

The avirulent organisms were obtained by serial transplantation of the virulent organism in homologous rabbit antiserum. To obtain the bacterial sediment for the respiratory systems the above conditions of growth, etc., were strictly observed.

The cultures were examined for purity and morphology. On the basis of previous studies the cultures containing more than 5 per cent of Gram-negative organisms were discarded.

The respiratory system consisted of 1 cc. 11/5 substrate solution, 2.5 cc. 11/15

Wieland, H., and Sevag, M. G., Ann. Chem., Berlin, 1933, 501, 151. Sevag, M. G., Z. Hyg. u. Insektionskrankh., 1933, 114, 756; Ann. Chem., Berlin, 1933, 507, 92; Biochem. Z., Berlin, 1933, 267, 211.

TABLE I

The Relation between the O<sub>2</sub> Consumed and H<sub>2</sub>O<sub>2</sub> Found\*

		C.mm. O <sub>2</sub> consumed		ท/100 2S2O₃	C.mm. O <sub>2</sub> recovered as H <sub>2</sub> O <sub>2</sub>		Per cent of O2 recovered as H2O2	
Pneumococcus	d-Glucose	Sodium lactate	d-Glucose	Sodium lactate	d-Glucose	Sodium lactate	d-Glucose	Sodium lactate
Virulent Type I (Laux) Young avirulent Type I (Laux) (avir-	865	1330	4.93	6.57	552	736		55
ulent for a week)Old avirulent Type I (Laux) (avirulent	840	1820	4.87	9.75	545	1092	65	60
since 1927)	650	1080	2.49	4.70	279	527	43	49
Virulent Type II (Erfurt)	1122	1192	5.33		597	628		52.7
Old avirulent Type II (Erfurt) (aviru-	1393	1238	8.18	8.17	912	915	65	74
lent since 1928)	500	986	1.78	2.63	199	295	39.8	30

<sup>\*</sup> The duration of the respiration period was 200 minutes.

TABLE II

Increase of O<sub>2</sub> Consumption in the Presence of Catalase and Sodium Pyruvate\*

		d-Glud	ose	So	Sodium lactate			(wi	piration thout a ostrate)
Pneumococcus	Alone	With CH3COCOONa	With catalase	Alone	With CH3COCOONa	With catalase	CH <sub>3</sub> COCOON <sub>a</sub>	With catalase	Pneumococcus alone
		. c.mm	G.mm	·	C.mm	C ****	-	c.mm	·
Virulent Type I (Laux)	1	2768	ı	1	i	î .	1	1	í
Young avirulent Type I (Laux) (avirulent		12.00	1	1			1		
for a week)	840	3585	9250	1820	3625	8300	437	450	84
Old avirulent Type I (Laux) (avirulent		ĺ	}	}	İ	İ	1		1
since 1927)	650	2280	3140	1080	3085	4080	143	280	55
Virulent Type II (Erfurt)	1122	2945	3750	1192	3055	5600	474	1482	199
Young avirulent Type II (Erfurt) (aviru-	1303	2928	5500	1220	2000	7619	1222	1324	69
Old avirulent Type II (Erfurt) (avirulent	1090	2920	3300	1230	3000	1040	1222	1324	0,
since 1928)	500	2534	2975	986	2246	2750	1589	1300	70

<sup>\*</sup> The duration of the respiration period was 200 minutes.

phosphate buffer pH 8.0 (or 1.5 cc. buffer with 1 cc. of catalase, or 1 cc. 11/5 sodium pyruvate solution), and 1.5 cc. bacterial suspension. To maintain the reaction at the original pH 8.0 we attempted to use M/8 phosphate buffer, but it was found that in this concentration of salt the organism undergoes marked and continuous autolysis, and hence it was found inadvisable to use a buffer solution stronger than 11/15. For the same reason a concentration of sodium pyruvate necessary for the complete removal of H<sub>2</sub>O<sub>2</sub>, namely M/2, could not be used. With every experiment the controls consisted of 3.5 cc. phosphate buffer (or 2.5 cc. buffer with 1 cc. of catalase or 1 cc. of 1/5 sodium pyruvate) and 1.5 cc. bacterial suspension.

TABLE III Percentage Increase of O2 Consumption\*

- cremiage	Tron.			
0	Increase of	0. c		
_	7	2 Consumi	Min.*	
Рлешпососсия	1		*10)[	
- Acting	d-(	lucose		
		-45006		
	Con With		Sodi	un lactate
Vi	CH3COCOON	a With		an include
Irulent Type T.C.		a catalase	CH-COSth	
Virulent Type I (Laux)	per cens		CHICOCOO:	With
Young avirulent Type I (Laux)  (avirulent for a week).  Old avirulent Type I (Laux)		per cent		catalase
Clavifulent for a spe I (Laur)	129	1	fer cent	
Old avirulent Type I (Laux) ulent since 1927) Virulent Type I (Laux) (avir-		469	i	fer cent
ulone wicht Type I (I	21.0	l	109	,
white since 1027/ (Laux) (avir.	316	1000		429
ulent since 1927). Virulent Type II (Laux) (avir- Virulent Type II (Erfurt). Young avirulent Type II (Fef.	1	1064		1
Young and II (Erfure)	260	1	84	1
avirulent Tyme		380		352
Young avirulent Type II (Erfurt)  (avirulent for a week)	168		100	
Oldaviruland week)	- 1	146	186	200
(avirulent Type II (Erfurt) Old avirulent Type II (Erfurt) Ulent since 1928) The durant	1	- 1	160 l	270
wient since 10201 (Effurt) (avie	29		1	315
1928)	- 1	215	1	
Thod	120	- 1	120	
the duration of the	120	200	- 1	441
As result of the respiration		290	. 1	
the acetic and a possible contation pe	riod van		T	
† The duration of the respiration per f As result of a possible combined et the acetic acid gradually accumulation obtained.	- "as 20	0 min		<i>5</i> 8
obtained. gradually accumulate	rect of lacta	uutes.		
dinulatin		IC. Direct		-

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The dry weight of bacteria (of 1.5 cc. bacterial suspension) used in all the experiments ranged from 8-12 mg. The results reported here, however, are recalculated for 10 mg. of dry weight for the purpose of immediate comparison.

The results in Table I show that during a 200 minute reaction period in the presence of glucose without a protective agent, the O2 consumed by four different organisms ranges from 650-1393 c.mm., and in the presence of sodium lactate from 986-1820 c.mm. The volume of O:

<sup>†</sup> As result of a possible combined effect of lactate, pyruvate concentration, and the acetic acid gradually accumulating, an accurate measurement could not be

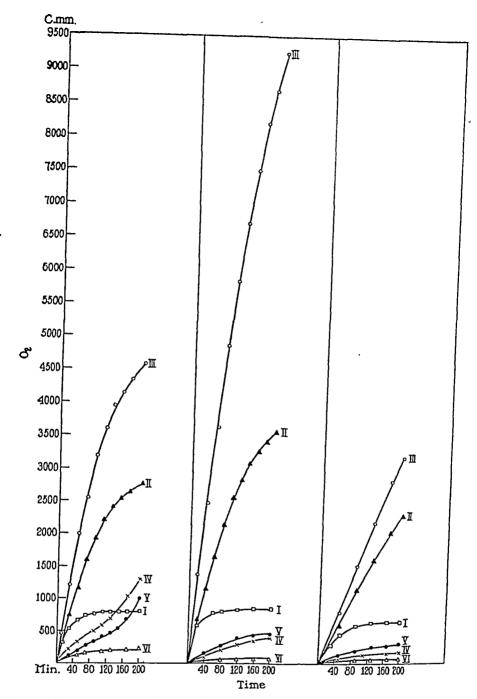


Fig. 1. Oxygen consumption during the oxidation of d-glucose by:

- (a) Pneumococcus virulent Type I (Laux).
- (b) Pneumococcus young avirulent Type I (Laux), avirulent for a week.
- (c) Pneumococcus old avirulent Type I (Laux), avirulent for a week.
- $-\Box$  = I, d-glucose alone + pneumococcus.
- $\blacktriangle = II$ , d-glucose + CH<sub>3</sub>COCOONa + pneumococcus.
- $-\circ -= III$ , d-glucose + catalase + pneumococcus.
- $-\times$  = IV, CH<sub>3</sub>COCOONa alone + pneumococcus.
- • = V, catalase alone + pneumococcus.
- $-\Delta = VI$ , pneumococcus alone.

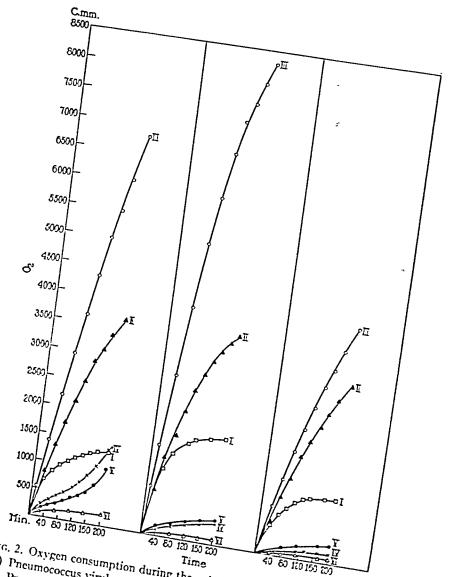


Fig. 2. Oxygen consumption during the oxidation of sodium lactate by: (a) Pneumococcus virulent Type I (Laux).

- (b) Pneumococcus virusent Type I (Laux), avirulent for a neek. (c) Pneumococcus old avirulent Type I (Laux), avirulent since 1927.
- C = I, sodium lactate alone + pneumococcus. II, sodium lactate + CII<sub>2</sub>COCOONa + pneumococcus.
- = 111, sodium lactate + catalase + pneumococcus. IV, CH<sub>2</sub>COCOONa alone + preumococcus.
- " V. catalase alone + pneumococcus.
- ~ VI, pneumococcus alone.

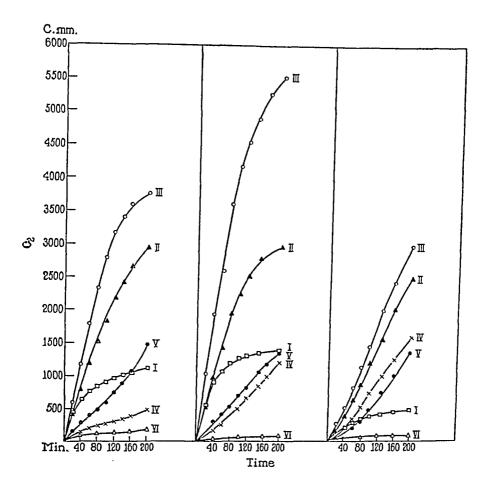


Fig. 3. Oxygen consumption during the oxidation of d-glucose by:

- (a) Pneumococcus virulent Type II (Erfurt).
- (b) Pneumococcus young avirulent Type II (Erfurt), avirulent for a week.
- (c) Pneumococcus old avirulent Type II (Erfurt), avirulent since 1928.
- $-\Box$  = I, d-glucose alone + pneumococcus.
- $\blacktriangle = II$ , d-glucose + CH<sub>3</sub>COCOONa + pneumococcus.
- --o- = III, d-glucose + catalase + pneumococcus.
- $-\times$  = IV, CH<sub>3</sub>COCOONa alone + pneumococcus.
- • = V, catalase alone + pneumococcus.
- $-\triangle --=VI$ , pneumococcus alone.

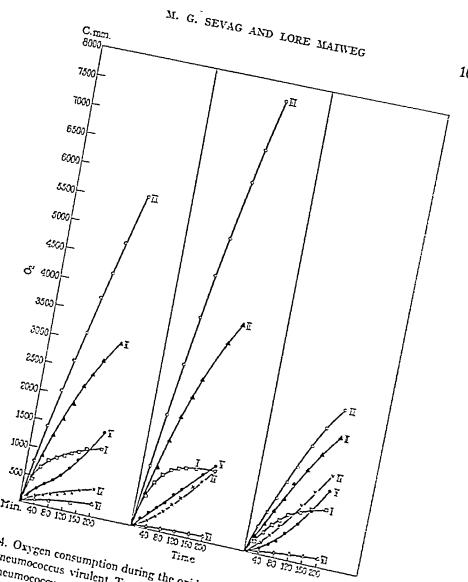


Fig. 4. Oxygen consumption during the oxidation of sodium lactate by: (a) Pneumococcus virulent Type II (Erlurt).

- (a) Pricumococcus viruient Type II (Eriurt).
  (b) Pricumococcus young avirulent Type II (Eriurt), avirulent for a week. (c) Pneumococcus young avaruaem. Type 11 (Esture), avaruaem 101 a

  1 costinui tostatoniona de manuscoccus, avirulent since 1928. A = II, sodium lactate + CII:COCOONa + pneumococcus. III, sodium lactate + catalase + pneumococcus.
- IV, CH2COCOONa alone + pneumococcus.
- Catalase alone + pneumococcus.
- VI, pneumococcus alone.

consumed by each organism, however, is neither a fixed quantity nor are the relationships presented constant. They vary within a certain limit from specimen to specimen, the differences in each series of experiments not exceeding 20-30 per cent. In the absence of a protective agent the ability of a given organism to use more oxygen than another depends on its ability to produce more pyruvic acid from glucose and lactic acid, its sensitiveness to hydrogen peroxide, and its resistance or tendency to autolysis. The same is true of different cultures of the same organism. These properties are often variable. For such reasons we are not able to make sharp differentiations. The results in the table show that with glucose 39-65 per cent of the oxygen consumed can be recovered as hydrogen peroxide, and with lactate 30-74 per cent. These values are by no means absolute. They vary from one experiment to another, but the variations are of negligible magnitude, and do not disturb the above relationships. It is to be noted that the ability of a virulent organism and its recently derived avirulent form to produce excess hydrogen peroxide is considerably greater than that of the old avirulent form of the same strain.

The addition of protective agents to the respiratory system brings about marked differences in the O2 consumption capacities of the various organisms. The most efficient protective agent is catalase, partly because of the fact that a small amount of a highly active enzyme preparation can be introduced without increasing the salt content of the system. It destroys two molecules of H2O2, yielding one The O2 so produced molecule of oxygen and two molecules of water. involves a liberation of energy which may be utilized by the cell. the other hand, in only a few cases does pyruvic acid approximate catalase as a protective agent. Its use is confined to a certain concentration, for the reason that a higher concentration brings about the autolysis of the organism. Consequently in the presence of insufficient pyruvate some H<sub>2</sub>O<sub>2</sub> is usually found in the reaction mixture. Furthermore the reaction products with pyruvate are acetic acid and CO<sub>2</sub> in the form of NaHCO<sub>3</sub>. These gradually inhibit or paralyze the Thus differences in organisms which, studied in a catalasecontaining system, are seen to possess markedly different activities,

may not be apparent when pyruvate is substituted for catalase. Nevertheless as a protective agent it has proved its usefulness by assuring the satisfactory growth of organisms such as old avirulent Type II (Erfurt) during a shorter period.

From an examination of Tables I to III, and Figs. 1 to 4 and the consideration of various observations, the following facts are evident.

A virulent pneumococcus on being transformed into its avirulent form consumes many times more oxygen than the parent organism; but this gain of activity is a temporary property. After a time it degenerates into a form which consumes very much less oxygen than either the virulent or the recently derived avirulent form. In a comparative study of the metabolic functions, and oxidation products of various virulent and avirulent pneumococci, these phenomena should receive consideration.

The change that takes place in the structure of the enzyme responsible for carbohydrate biosynthesis during the shift from the virulent to the avirulent form may be associated with the changes in the enzyme structure already demonstrated in connection with these



## STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF RABBITS

## I. TRANSMISSION AND FILTRATION EXPERIMENTS

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(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, April 16, 1934)

In previous articles (1, 2) we have described spontaneous conjunctival folliculosis as it occurs in Macacus rhesus monkeys and in chimpanzees. This condition, prevalent among certain dealers' stocks of animals (1, 3), was identified as a disease sui generis, which is localized wholly to the conjunctiva and is transmissible from simian to simian by contact or by the inoculation of affected tissues. It was found to be due to a bacterium, not to a virus, and a new organism, tentatively designated as Bacterium simiae, was shown to have an intimate relationship to the infection.

A spontaneous conjunctival folliculosis exists widely distributed in various species of rabbits: chinchilla, New Zealand and domestic, either albino, black or pied. For example, in two groups of animals, 52 in one and 129 in the other, only six of each group had smooth conjunctivae; and again, inspection of rabbits in dealers' stocks revealed that of 115 animals, only about 25 per cent, consisting chiefly of the suckling or the newly weaned, and those born of mothers with smooth lids, were free from this conjunctivitis.

The occurrence of folliculosis in rabbits has been recorded by others. Weiss stated that follicles have been seen in "normal" animals by Finnoff and Thygeson, Bengtson, and himself (4). Cuenod and Nataf (5) mentioned that the affection occurred frequently and classified it as "pseudotrachoma." The writers cited have not reported any experimental investigations.

A laboratory study of the condition was, however, made by Nicolle and Lumbroso (6). They termed the rabbit affection conjunctivite granuleure naturelle and

described two forms: one in which follicles were massed at the canthi, the follicles being considered as normal (follicules normaux); and the other in which the lesions were scattered over the entire lid. In either case the upper, tarsal, conjunctiva was always clear. This absence of lesions in the upper lid was considered by them as a differentiation of the rabbit disease from human trachoma, since they could determine no distinction in the type of follicles in both affections.

Nicolle and Lumbroso also found that the disease was transmissible from rabbit to rabbit either by inoculation of affected conjunctival material or by contact; the new-born being frequently attacked as a result of contact with infected mothers. They reported, furthermore, that rabbit folliculosis was transmissible to monkeys (Macacus sinicus and rhesus) and a papion (baboon). A single animal of each species was employed for this test. Although they concluded in a first article (6) that Macacus inuus monkeys were not reactive to inoculation of rabbit folliculosis material, they reported in a second paper (6) that two inuus monkeys had been infected with such material, the effects, however, differing from those in the other simians. The period of incubation was at least 10 weeks, instead of 2, and the lesions appeared in both the upper and lower lids, instead of only in the lower. However the recognition of the specificity of the reaction in the inuus animals is important, since the writers asserted that isolated follicles appeared spontaneously among their monkeys, and that in their hands mere scarification of conjunctivae harboring such lesions induced an intensified folliculosis over the These aspects of the problem as they concern spontaneous entire membrane. granular conjunctivitis of monkeys have already been discussed in one of our previous papers (1). Nicolle and Lumbroso believed that there might be a telluric or humic origin of the rabbit disease (des maladies telluriques ou mieux humiques). In their opinion, the fact of its spread by contagion from rabbit to rabbit does not render untenable the view that the origin of folliculosis is in the soil or humus with which the animal comes in contact.

After completion of the investigation on spontaneous follicular conjunctivitis of *rhesus* monkeys and chimpanzees, with results as given in the introductory paragraph of this paper, we began a similar study of rabbits, with the object of determining the etiology of the malady as well as any possible relationship of the conjunctivitides among different species. In the present paper, we shall describe the clinical and pathological appearance of the disease and the attempts to identify the nature of the causal agent. In a second article we shall report bacteriological investigations and discuss the significance of the results.

#### Clinical Obscrvations

We have found, like Nicolle and Lumbroso (6), that the conjunctivitis as it occurs in rabbits exists in two forms, which we designate

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respectively as the localized (Type I) and the diffuse (Type II). either case, were it not for the disclosure of the lesions after eversion of the lids, and occasionally for the presence of abnormal secretion at 109 the inner canthus or the caruncular region of the eye, the rabbits could be considered as wholly normal.

The Localized Form of the Disease.—In Type I of the spontaneous disease eversion of the lids discloses apparently normal conjunctivae except for an occasional case in which there is a mild degree of edema with injection of the vessels descending from the palpebral arterial arch. A closer examination, however, reveals at the canthi, more often the inner, three or four follicles in vertical alignment, like a chain of beads, or they may be irregularly massed, in a minute, hummock-like clump. The latter consists of four or five follicles of different sizes, the entire clump measuring from 2 to 3 mm. across the widest part. One or two isolated, small follicles may appear also in the mid-section of the conjunctiva. While Nicolle and Lumbroso have found the upper lids to be always free from lesions, we have observed, on the contrary, that about half of the animals examined reveal follicles situated in the upper palpebral conjunctive in positions similar to those in the lower. In the upper membrane, however, the more frequent congestion and mild edema may mask the granulations. Considerable aid in such instances is afforded by the use of an ophthalmologist's loupe.

The essential lesion in the first form of the disease is the follicle. It appears as an elevation of minute, pin-point size which, discrete and located in the mid-section of the membrane, may measure up to 1/2 mm. in diameter. In general, the follicles resemble semitransparent, gelatinous sago-like granules, light greyish in color, with an even, smooth surface. Pressure between glass slides ruptures them, expressing a greyish, grumous substance which on microscopic examination will be seen to consist of cellular elements, notably large epithelial cells and monocytes. The follicles are never dark red and hemorrhagic, as are those occasionally met with in monkey folliculosis. The bulbar conjunctiva and the comea are not involved in any way: there are no visible scars, pannus, ulcers or pits. The follicles may be transitory and disappear after several weeks but remission with newly formed lesions is the rule. In some animals the affection remains stationary for indefinite periods; in others more marked activity results in the development of all of the characteristics of the diffuse form of the disease. We shall describe later

The Diffuse Form of the Disease.—Type II of rabbit conjunctival folliculosis, although less often seen in nature than Type I, is more persistent and is usually associated with a progressive inflammatory reaction. While the type of follicles is similar in both forms of the disease, their distribution is different. In Type II the lower conjunctiva is also more markedly involved than the upper. The mucous membrane, dark red in color, presents injected blood vessels and a surface roughened and thickened by the underlying edema. It is covered in its entirety

by granulations of various size which may lie in parallel rows or be irregularly distributed. Here, as in Type I, the upper conjunctiva is also affected and reveals more marked congestion and edema with a resultant velvety appearance. Characteristic follicles, although scattered over the surface and especially at the inner canthus, are less numerous than those in the lower conjunctiva. There is often present a drop of greyish or faintly yellowish, mucopurulent secretion at the inner angle of the eye.

This description applies to the form of disease as it is ordinarily seen in nature. After the disease has become chronic, the entire lower membrane becomes studded with uniform sized granulations, not unlike a gravel stone mosaic in appearance. In such instances the membrane assumes the greyish color of the massed follicles. Pannus of the cornea, ulcers, pits or visible scars also do not develop in this form of the disease. In most of the animals an ovoid network of injected blood vessels, measuring about 5 mm. in the longest axis, is present in the bulbar conjunctiva at the upper pole of the corneal limbus.

In the diffuse type of spontaneous rabbit folliculosis, the disease lasts, as a rule, throughout the life of the animal. It is, however, essentially progressive, taking several weeks, or months, to reach a stage of stationary chronicity. During the progress of the affection infrequent exacerbations or remissions may nevertheless occur, but the general tendency of slow progression to chronicity with a fully developed reaction remains. Figs. 1, 2 and 2a show the lesions in the upper and lower conjunctivae in fully developed cases.

Relation of Type I to Type II of the Disease.—The question whether the lesions of the localized form of folliculosis are simply enlargements of pre-existing conjunctival lymphoid tissue (6), or whether they constitute another form of the same disease is answered by the results of the following experiments.

Two series of tests were undertaken. In one, 25 stock rabbits showing characteristic Type I folliculosis and kept under quarantine were observed for the purpose of noting the progress of the disease over varying periods of time. Ten of the animals developed, during the natural course of the affection, in the 4th to the 6th week of observation, the diffuse or Type II form of folliculosis.

In the second test, each of three anesthetized rabbits having Type I conjunctivitis with lesions in all four lids was scarified in the upper left membrane only, by means of stippling with a No. 22 hypodermic needle. From 12 to 20 days later the animals revealed the diffuse type of folliculosis in the scarified conjunctiva,

<sup>&</sup>lt;sup>1</sup> All experimental procedures on animals as reported in this paper were performed with the aid of ether anesthesia.

thence spreading during a period of 2 to 3 weeks from this lid to the other, untreated, membranes. As controls, fourteen rabbits having smooth conjunctivae were similarly scarified; none of these was specifically affected. 111

At this point it may be mentioned that, of over 50 rabbits with definite, characteristic Type II folliculosis, four showed retrogression of the lesions; after a month it became clearly evident that the disease took on the form of the localized type. This, as we have already stated, is an unusual occurrence; the diffuse form of folliculosis is, as a rule, progressive and persistent.

It would appear from the foregoing that the Type I affection is an expression of a mild form of the malady, and the diffuse type of a relatively marked form, one being convertible into the other by natural or artificial means. Whether the type of folliculosis which develops in an animal is correlated with the degree of its resistance or with environmental factors is unknown.

The results of microscopic examination in the two types of the disease offer additional evidence that both consist essentially of varying degrees of the same pathological changes. In Type I the lesions, chiefly follicular, are limited to only part of the conjunctiva, the remainder of the membrane being normal; in Type II, the entire lid is involved but with the same kind of follicular reaction (Figs. 3, 4, and 5). A description of the lesions existing in the diffuse form should

The epithelium loses its continuity; here and there are observed more or less extensive areas of complete denudation of the membrane or its thinning out to a single cell layer, especially over a superficially placed follicle. Papillary hypertrophy does not occur; nor do the pseudocystic formations, known as post-trachomatous degenerations (concretions formed in epithelial pseudocysts due to papillary hypertrophy). In areas in which the follicles do not protrude beyond the lining epithelial zone, there is a mild degree of infiltration mainly with lymphocytes, but also with monocytes and polymorphonuclear leucocytes containing

The follicles are either discrete or confluent and develop in the more superficial layers of the conjunctiva. They consist chiefly of cells having clear homogeneous cytoplasm and vesicular nuclei without much chromatin. These clasmatocytes are more abundant in the center of the lesion in which may also be found lymphocytes and an occasional plasma cell. The lymphocytes show, however, a more pronounced disposition towards the periphery; but the orderly arrangement of a

definite center of clasmatocytes surrounded by a well defined zone of lymphocytes, such as is seen in other conjunctival follicular diseases, does not occur in rabbit folliculosis. A slender, fibrous capsule encircles the follicle. The blood vessels throughout the conjunctiva are dilated and distended with blood. In general, the lesion appears superficially like a granuloma, but granulation tissue and giant cells are absent (Fig. 6). On the other hand, bacteria are often seen, lying either free or intracellularly in the follicle. These will be decribed in the next paper of the series. We should state here, however, that Bacterium granulosis or simiae was not recovered from the affected tissues.

The cellular infiltration in the case of follicles that are in a relatively earlier stage of development is predominantly lymphocytic, although monocytes, primitive connective tissue cells and a few clasmatocytes are also visible; but encapsulation is not evident (Fig. 7).

#### Diet as a Causal Factor

In view of the fact that dietary insufficiency has been advanced as the causal factor of conjunctival folliculosis (7), an attempt was made to find whether an insufficient diet would cause the rabbit disease.

Nine rabbits having smooth conjunctivae and in an apparently healthy condition were quarantined. One or all of the conjunctivae were scarified once at the beginning of the test and then the animals were placed on a diet limited solely to oats. They rapidly became thin, the average loss of weight after 34 days being 483.3 gm. from an initial average weight of 2039 gm. None developed follicular conjunctivitis.

Restriction of the diet to oats did not give rise to folliculosis. Tests now to be described indicate that the disease can be conveyed from rabbit to rabbit by contact or by inoculation of folliculosis tissues. This being so, it is difficult to assume that the rabbit disease is telluric in origin (6) or that it is an expression of generalized adenoidism (8).

#### Transmission Experiments

(a) By Inoculation of Affected Tissues.—The entire affected conjunctivae of anesthetized rabbits having spontaneous folliculosis were removed and ground in a mortar with 0.9 per cent sodium chloride solution, in proportion of 1 cc. of the solution to four conjunctivae; the resulting tissue suspension was injected into the left lower conjunctiva and was also applied to the scarified conjunctivae of normal rabbits. In general, the methods used followed those of Noguchi (9) in his studies on trachoma

For 3 or 4 days after receiving the affected tissue, the conjunctiva of the rabbit shows the acute, irritative effects of the inoculation and scarification; namely, congestion, edema and roughening of the membranes. This acute condition sub-

P. K. OLITSKY, J. T. SYVERTON, J. R. TYLER sides promptly and after another day or two, or 5 to 7 days after injection, a definite follicular conjunctivitis develops. The early stages reveal a membrane roughened in appearance with minute granulations especially at the inner and outer canthi and on the semilunar fold. From these marginal areas, the follicular reaction spreads over the entire palpebral conjunctiva in a period of from 1 to 3 weeks. Meantime the uninoculated conjunctivae become involved with the same type of inflammation, and within about a month all of the palpebral membranes are affected. The experimental disease is then indistinguishable from the affection occurring in nature. Infrequently the period of incubation of experimental folliculosis is prolonged to 2 or 3 weeks. Abscess formation does not occur following inoculation.

The following summarizes numerically the results of the tests. The tissues were obtained from 20 rabbits having characteristic Type II folliculosis. As a rule, the material from two animals was pooled before injection. It Was inoculated into the conjunctivae of 22 rabbits in all, two or three receiving each material. One of the 22 died early, one (of a group of three) was unaffected, and the remaining 20 reacted in the manner just described and developed the diffuse form of the disease. In one series of tests the affection was initiated in two rabbits having smooth conjunctivae by means of the subconjunctival injection of folliculosis tissue derived from a stock rabbit. By this means the disease has been transferred through six passages to date—each passage consisting of two rabbits with Positive results in all. Fig. 5 represents the lesions induced in one of the On the other hand, conjunctival material showing localized folliculosis (Type I)

Seldom gave rise to the diffuse form of the disease. Thus, only one of three rabbits having Type I folliculosis evoked the diffuse form in two normal animals. Transfer of such material from the other two affected rabbits showed no specific effects in four normal animals.

The inference that the rabbit is highly sensitive to the agent causing diffuse conjunctival folliculosis is justified, since all but one animal showed characteristic diffuse folliculosis after injection with affected tissues. The agent producing the localized form of the disease proved to be less active.

(b) By Instillation of Suspensions of Affected Tissues.—The scraped follides derived from two rabbits with the Type II disease were suspended in 1 cc. of saline solution and such a suspension was freshly prepared for use on each of a consecutive days. This was dropped into the conjunctival sac of three normal rabbits with Eastle massage for some seconds. From 5 to 11 days after the last instillation, signs ef folliculosis appeared and in 2 or 3 weeks developed into the characteristic, diffuse type. The same material inoculated subconjunctivally into two additional rabbits, as controls, induced diffuse folliculosis in both within a week.

(c) By Contact.—Two stock rabbits showing spontaneous diffuse folliculosis were placed in the same cage with two rabbits having smooth conjunctivae. After 15 days, both of the latter showed characteristic Type II folliculosis. As controls, three animals with clear conjunctivae were caged together; during 5 weeks' observation none was affected. The tests were repeated with the same outcome. The extension of the disease from the inoculated conjunctivae to the uninoculated membranes in other experiments, as already mentioned, is another example of contact infection, autogenous in origin.

The conjunctival folliculosis occurring spontaneously in rabbits is manifestly an infectious disease. It is not only transmissible by means of inoculation or even instillation of a suspension of the affected tissues, but it is also contagious: animals with smooth conjunctivae acquire the infection when merely caged with folliculosis rabbits.

The fact that the disease can be spread by contact emphasizes the necessity for rigid precautions during experimental work. For this reason, as well as because of the widespread distribution of the disease in nature, the choice of animals for such work is difficult. The best material is provided by the 2 or 3 month old litters of does free from conjunctival affections in that they are least likely to have the spontaneous disease. Animals are kept, as a rule, from 3 to 5 weeks in strict isolation and quarantine before use. In spite of these precautions, five of 204 animals developed localized follicular conjunctivitis during the early stages of quarantine.

Control Experiments.—The following control experiments would seem to indicate that rabbit folliculosis and the induced disease are a clinical entity resulting from a specific infectious agent.

In fourteen anesthetized animals the palpebral conjunctivae were merely scarified; in nine, the membrane was scarified and normal rabbit conjunctival tissue applied to it; and in nine, subconjunctival inoculation of similar material was made in addition to its application to the scarified tissue. None of these 32 rabbits developed folliculosis.

Twenty-eight animals were inoculated subconjunctivally with various bacteria encountered in cultures of conjunctival tissue obtained from monkeys or rabbits with or without folliculosis; and in addition, fourteen with cultures of either Bacterium granulosis or Bacterium simiae and seven with suspensions of sterile leptospira medium. Characteristic lesions were not produced in any of these animals.

Six rabbits received suspensions of human trachomatous tissues with negative results.

Suspensions of the conjunctivae of three rhesus monkeys showing characteristic spontaneous folliculosis (1) proved inactive on injection into the conjunctivae of six rabbits. Conversely, the tissue from the affected lids of six rabbits having 115 fully developed folliculosis, which in each case evoked the experimental disease in other rabbits, failed to infect four Macacus rhesus and three Macacus cynomolgus monkeys. The issue of this last experiment adds to the list of control tests and does not accord with the finding of Nicolle and Lumbroso (6) that the rabbit affection is transmissible to monkeys.

### Filtration Experiments

After the specific, infectious nature of spontaneous conjunctival folliculosis of rabbits was determined, we undertook a study of the filtrability of the causal agent through Berkefeld V candles or Seitz membranes. Cultures of Serralia marcescens (Bacillus prodigiosus) could pass through the former but not through the latter.

In all instances hormone broth, which favors the filtration of viruses and bacteria, was employed as diluent. The mucous membrane of the conjunctivae removed from rabbits having fully developed folliculosis was ground in hormone broth, 5 cc. being added to tissue derived from two to four lids. The thin suspension so obtained was allowed to stand in order to sediment the larger particles, and the supernatant fluid was then placed in a Seitz apparatus or in a Lilliput, size No. 5 Berkefeld V filter, the candle of which was covered with a fairly close fitting, inverted test tube so that nearly all the material could be passed through. Filtration was done at a negative pressure of 60 cm. Hg, and continued for about 10 minutes, the time required to obtain sufficient material for experimental purposes. The filtrate was seeded in leptospira medium but in no case was growth

Seven separate tests were made; in four, small size Berkefeld V filters were used, and in three, a single Scitz disc. In all but one experiment the affected conjunctivae obtained from two rabbits were pooled to prepare the suspension to be filtered. The unfiltered and filtered material was inoculated into two rabbits for

To summarize: In the seven tests all rabbits, fourteen in number. receiving unfiltered folliculosis material developed the characteristic experimental disease of the diffuse type. Of the corresponding fourteen animals injected with filtrates, thirteen were unaffected and only one showed folliculosis. The question may well be raised of whether this rabbit became infected accidentally through contact with the active agent of the disease.

It can be said, therefore, that the infectious element does not, as a rule, pass through Seitz filters which retain *Scrratia marcescens*, or through Berkefeld V filters which are pervious to this organism.

Absence of Inclusion Bodies.—In view of the fact that filtrability by itself is not always an indicator of the character of an ultramicroscopic virus (10), we extended the study to include an examination of stained film preparations and sections of tissue from folliculosis cases, for the peculiar cytotropic effects which are characteristic of the action of many viruses (11).

Twenty-two rabbits showing fully developed folliculosis in early and late stages were examined by means of Giemsa-stained film preparations of follicular material. Two to four such slides were made from each rabbit and the microscopic examination included the contents of the entire film. In addition, sections of affected tissues removed from over 25 animals and stained with eosin and methylene blue or by the Giemsa method were studied microscopically. We could not find in these film preparations or tissue sections, either intra- or extranuclear inclusion bodies which might be referable to the action of an ultramicroscopic virus. Nor were the primary cellular changes caused by various viruses observed.

The evidence as given indicates that an ultramicroscopic virus is not the cause of the disease.

#### CONCLUSIONS

Spontaneous conjunctival folliculosis is widespread among various species of rabbits. It exists in two forms: Type I, in which the lesions are localized and the disease is relatively inactive, and Type II, in which the follicles are closely distributed over the entire surface of the conjunctivae and the affection is more active and characterized by extensive inflammatory reactions. One type can be converted into the other either by experimental methods or by natural processes.

The disease can be transmitted from rabbit to rabbit by means of subconjunctival inoculation of suspensions of the affected tissues or by instillation of such material into the conjunctival sac, or even by mere contact of folliculosis animals with rabbits having smooth conjunctivae. It is plain that the disease is an infection.

The causal agent of the infection is not filtrable through Seitz discs that retain Scrratia marcescens nor through Berkefeld V candles that permit the passage of this organism. Furthermore, the lesions of the

spontaneous or of the experimental disease do not exhibit the cytotropic effects or the inclusion bodies suggestive of the action of an ultramicroscopic virus. They are characterized, on the other hand, by a persistent and progressive chronicity and show certain resemblances to the granulomata.

The evidence suggests that the spontaneous conjunctival folliculosis of rabbits is due to a microorganism—one having a low grade pathogenic action. In a paper shortly to be published, a bacterium capable of reproducing folliculosis in normal rabbits will be described.

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#### EXPLANATION OF PLATES

All sections are stained with hematoxylin and eosin, except Fig. 4 which is stained with eosin and methylene blue.

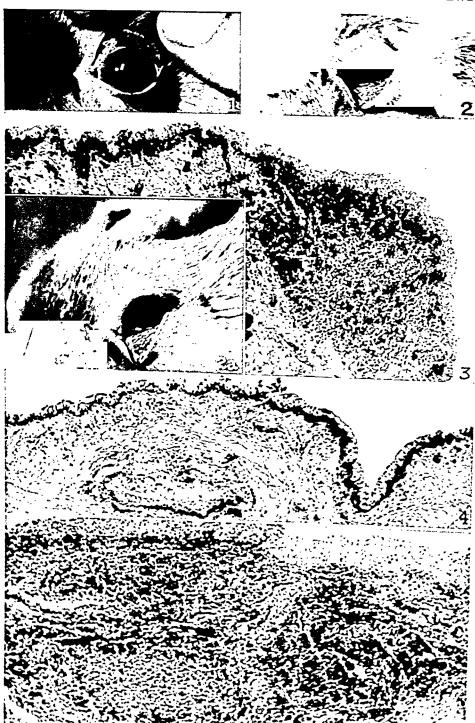
#### PLATE 4

- Fig. 1. Eye of stock rabbit having fully developed diffuse conjunctival folliculosis (Type II). The upper lid shows the velvety appearance of the conjunctiva, associated with numerous follicles. Natural size.
- Fig. 2. Same rabbit, lower lid. Granulations diffusely scattered over the entire conjunctival surface. Natural size.
  - Fig. 2 a. Same as Fig. 2, in another rabbit.
- Fig. 3. Localized conjunctival folliculosis (Type I) in a stock rabbit. The solitary follicle is to be noted. The remainder of the conjunctiva was practically normal (as shown in Fig. 4).  $\times$  122.

- Fig. 4. Same as Fig. 3. The conjunctive is normal in the portion free from follicles.  $\times$  122.
- Fig. 5. Rabbit conjunctiva, inoculated with affected tissue, showing diffuse folliculosis Type II. There is a marked follicular reaction with widespread inflammatory changes.  $\times$  122.

#### PLATE 5

- Fig. 6. Same specimen as Fig. 5. A higher magnification of the follicular structure which consists of light staining clasmatocytes, some lymphocytes and plasma cells. A fine fibrous capsule can be seen to be limiting the follicle. × 300.
- Fig. 7. A follicle in its earlier stages of development; Type I folliculosis. One sees monocytic infiltration with lymphocytic accumulation, at the margin more especially. Fibrous capsule absent. × 300.



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# THE ANTIGENIC RELATIONSHIP BETWEEN PROTEUS

II. A STUDY OF THE COMMON ANTIGENIC FACTOR (From the Department of Bacteriology and Immunology, The Harvard Medical

In a preceding paper, the writer with Zia (1) reported upon the antigenic relationship between B. proteus X-19 and typhus Rickettsia. It was shown that when suspensions of Rickellsiae were prepared in the usual way, they were not agglutinable in anti-Proteus serum. However they became agglutinable when formalinized and heated at 75°C. for 30 minutes. This phenomenon suggested that formalin preserves a certain Rickellsia agglutinogen, which, under ordinary conditions, disintegrates. At the same time the formalin modifies the Suspensions of Rickellsiae in such a manner that unless these suspensions are heated, agglutination does not occur. By the Castellani technique it was shown that both organisms acted towards the homologous and the heterologous sera in a manner similar to that which is observed in the case of some bacteria where the antisera have two types of agglutinins, one so called major agglutinins, specific for the homologous organism, and the other, minor agglutinins responsible for the agglutination of the heterologous organism. The crossagglutinability of Rickellsia and Protens X-19 by typhus immune serum and anti-Proteus serum showed that there is a common antigenic factor in both organisms which, in typhus fever, develops agglutinins for Proleus X-19 and, in animals immunized with Proleus X-19, agglutinins for Rickellsia.

The present paper is a report of attempts to establish the probable nature of the common factor in Rickellsia and in Proteus X-19. Efforts to obtain significant antigenic substances by extraction of Proteins were without result. We therefore concentrated our efforts

upon the extraction of carbohydrate materials analogous to those obtained from pneumococci and other bacteria.

Such polysaccharides have been obtained by Przesmycki (2) from the H and O forms of B. proteus X-19, but his OX substances were of low precipitating titre. Furth and Landsteiner (3) obtained precipitable substances from Proteus X-19 which reacted like the non-protein-precipitable substance of B. typhosus and contained a large proportion of carbohydrates. Lim and Kurotchkin (4), by repeated alcohol precipitations from alkaline extracts of Proteus X-19, prepared substances which gave specific precipitation and complement fixation when treated with human typhus serum.

#### Methods

In preparing our own extracts we used two methods as follows:

- 1. To a 5 per cent solution of antiformin a thick suspension of organisms from solid culture media was added, and after shaking for 30 minutes, glacial acetic acid was added until a heavy precipitate formed. After centrifugation the supernatant fluid was dialyzed and concentrated to one-eighth its volume at 60° to 70°C. The concentrate was then centrifugalized and the supernatant fluid precipitated in the cold with alcohol and then treated with ammonium sulfate, by methods analogous to those used by Avery and Heidelberger (5) for the production of pneumococcus polysaccharides.
- 2. 8 day broth cultures of *Proteus* X-19 were concentrated to one-tenth their volumes. The concentrate was precipitated with one volume of alcohol which after 1 hour standing, brought down impurities and left the antigenic fractions in the supernatant fluid. To this fluid, alcohol was added. After 24 hours in the cold, a precipitate formed which was then purified by fractional alcohol precipitations.

The materials obtained by the antiformin treatment were lower in nitrogen content (7 per cent) than were those obtained by the broth method. All preparations gave powerful Molisch reactions.

The X-19 soluble specific substance is heat-stable and withstands boiling in acid as well as in moderate alkaline reaction; but alkali, even at room temperature, seems to cause deterioration of the product on long standing.

The X-19 extracts when tested with *Proteus* X-19 antiserum gave precipitations in dilutions of one to five million. Such extracts give definite precipitations with typhus convalescent serum as well as with the serum of a horse treated by intravenous injections of formalinized Mexican *Rickettsiae*.

# Rickettsia extracts were prepared by the following technique.

Formalinized Mexican Rickettsiae from X-rayed and infected rats were washed twice in 0.2 per cent formalin in salt solution, then treated with ammonium hydroxide to a concentration of 0.5 per cent and left overnight at room temperature. They were then treated with 4 per cent antiformin for 3 to 4 hours and centrifugalized. The supernatant was neutralized with acetic acid and stored in the ice chest. The sediment was resuspended in water and treated again with 2 per cent antiformin and left overnight at room temperature. It was again centrifugalized after neutralization with acetic acid. A microscopic examination of the sediment was made which did not show any Rickellsiae. The sediment was washed once more in water and the pooled supernatants were dialyzed for 5 days, after which the fluid was centrifugalized and concentrated by evaporation in the water bath to the original volume of the vaccine used in the preparation. Enough sodium chloride was added to make a concentration of 0.85 per cent.

The Rickettsia extracts are heat-stable, giving a negative biuret and a positive Molisch test. The purification of such extracts was not possible owing to the extremely small amounts of material available. Nevertheless the positive Molisch test is very significant of a carbohydrate nature of the active factor considering that the vaccine undergoes two washings in formalinized saline which would eliminate practically all the glycogen from the rat serum.

The Rickellsia extracts, when mixed in even amounts with a 50 per cent diluted typhus human serum or Proleus X-19 antisera, gave a definite precipitation which was easily read after incubation of the mixtures at 45°C. for 3 hours and better after standing overnight in the ice chest. A considerable number of controls were made. Table I is an example of the cross-precipitations.

The experiment presented in Table I gives evidence of the presence of a soluble specific factor in the Rickellsia bodies, which reacts in the same manner as the OX-19 extracts when mixed with typhus serum and anti-Proteus serum. It is not unlikely to be a polysaccharide of the same nature as those extracted from B. proteus OX-19.

The experiment, together with the precipitation experiments of X-19 soluble specific substance, satisfy us as to the identity of an antigenic fraction in both Rickettsiae and Proteus X-19, but the following experiments add a further evidence in corroboration.

TABLE I

Precipitation of Proteus X-19 and Typhus Scra with X-19 and Rickettsia Soluble
Substances

Sera•	X-19 extract	Rickettsia extract
Anti-Proteus X-19.	+++	+++
Normal Rabbit 1		\ <del>'</del> '
Normal Rabbit 2	( – !	_
Normal Rabbit 3		_
Normal Rabbit 4		
Mexican human Typhus IV†	++	++
Mexican human Typhus VI†		++
Normal Human 1†	_	-
Normal Human 2†		
Normal Human 3†		-

<sup>\*</sup> Sera were centrifugalized at high speed and diluted 50 per cent in saline.

TABLE II

Absorption of X-19 Antiscrum by X-19 SSS

	Agglutina	tions with	B. proto	us X-	-19			
Serum dilutions	1/40	1/80	/80 1/16		1/320	1/640	1/1280	1/2560
Absorbed serum Non-absorbed serum	++++	+++	+ + + +	 ++ ++	++++	+++	++	+
Pre	cipitations v	vith X-19	SSS (1/0	500 di	lution)*			
Serum dilutions	1/2		1/4	1/8		1/16		/32
Absorbed serum Non-absorbed serum	+++-	- +-	- +++	- +++		- +++	-	_  -  -
Agglutinati	ons with hea	ted form	ılinized N	1exica	ın <i>Rickellsi</i>	ae		
Serum dilutions	1/40		1/80 1/160		60	1/320		
Absorbed serum Non-absorbed serum	++		- +		- +	·		

<sup>\*</sup> Precipitation tests by the ring method.

<sup>†</sup> This serum was preserved in 0.25 per cent carbolic acid.

As stated in our paper with Zia (1) the Rickettsia bodies absorb the agglutinins for Rickellsia as well as those for Proteus X-19 from typhus serum; while Proteus X-19 absorbs only the homologous Proteus agglutinins from typhus serum. From Proteus X-19 antiserum ab-

TABLE III Typhus Scrum Absorbed by X-19 SSS

Typhus San	1
Typhus Serum Absorbed  B. Proteus V. 10	1. **
P	oy X-19 SSS
Serum dilutions 1/40 J	1
1/40 1/60 1	Tr
1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	Weigl European vaccine
3 boot 1/320 1/640	
absorbed se-	1 1/100 1 1/2-1
rum	1/320 1/640
1) phus human   -   -	
SCIUM Contral	
	***   **   **
absorbed the state of the state	+   +   +
THE SOLUED CALL IN THE LAND CONTRACT OF THE LAND CO	+++
rum	++   +
Alorse immune	'   +
Serum cont       -   -	.
	+   ++   +
* Very 6- ++++ ++++ ++++	1 1 + 1
† Doc : nne granular, but de la tra	14   1   1   1
Dennite floccular, but definite application	
* Very fine granular, but definite agglutination.	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1

Absorption of	of D	TARLE -		
Antigen	7 Poleus X-19 Anti	scrun: by tra-		
Serum dilutions.	Y-19 SSS (precipitati	ons)*	can Formalinized	Rickellein
Anti-Proteur 3	1/4 1/8 1/1	16 1 1	Freieus X-19 (aggluti	national .
serum absorbed		1/40	1/80 1/160	Limit
Anti-Proteur 3-	#   -   -	]_ [.		1/320 1/640
10 scrum con-			+++++++	l, . l
* Precipitation	-+++ ++++ ++	+ + + +		+++  -
sorption with a	test by the ring	$\frac{1}{\text{od}}$	+++ ++++ -	+++1,
"O'PHON With a		· (4,		・ナーナ

sorption with homologous organisms takes out the agglutinins for both Proleus X-19 and Rickellsia: while Rickellsia bodies take out only the agglutinins for Rickellsia. In Table II are shown the results of the absorptions of Proteus X-19 antiserum with X-19 SSS.

The experiment given in Table II demonstrates that the anti-Proteus serum has two antibodies, one which is responsible for the precipitation of the soluble specific substance of the Proteus X-19 and causes agglutination of Rickettsiac. The other antibody, which remains in the serum after absorption with X-19 SSS, is still capable of agglutinating Proteus X-19, but has no effect on Rickettsiac. On the other hand, if typhus convalescent human serum, or immune antityphus serum from a horse is treated with X-19 SSS, the results parallel those obtained by the absorption of such sera by Proteus X-19 organisms, as shown in Table III.

Finally, to complete the experiments presented in Tables I to III, it was considered advisable to determine whether the serum of rabbits treated with *Proteus* X-19, and adsorbed by the extracts obtained from *Rickettsia* bodies would act in a similar manner, but it was not feasible to produce *Rickettsia* extracts in amounts sufficient for the experiment. For this reason, an indirect method of investigation was devised. *Proteus* X-19 antiserum, diluted from 1/4 to 1/20 in different experiments, was absorbed with *Rickettsia* bodies, until no more agglutination for heated *Rickettsiae* was detected. Then the absorbed serum and dilutions of the control serum, were tested for precipitins with X-19 SSS. The results are seen in Table IV.

This experiment shows that anti-Proteus serum absorbed with Rickettsia loses its antibodies for the supposedly common SSS factor but still contains agglutinins for that part of the Proteus X-19 antigenic complex not represented in the Rickettsia.

### SUMMARY AND CONCLUSIONS

A soluble specific substance was isolated from Mexican typhus *Rickettsia* which gave, with *Proteus* X-19 antiserum and typhus human serum, the same precipitation reactions as the polysaccharides extracted from *B. proteus* OX-19.

The soluble specific substance extracted from *Rickettsia* and *Proteus* OX-19 is likely to be of a polysaccharide nature owing to the strong Molisch reactions obtained with such extracts, the heat stability and the negative protein reactions (biuret). Since, however, it still contains 7 per cent nitrogen, this is not certain.

In the antigenic composition of both Proteus X-19 and typhus

Rickellsia there is a common soluble specific factor which is responsi-125 ble for the Weil-Felix reaction.

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# AN IMMUNOLOGICAL STUDY OF THE A SUBSTANCE OR ACETYL POLYSACCHARIDE OF PNEUMOCOCCUS

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In 1930 (1) one of us described a type-specific substance in the autolysates of Pneumococcus Type I which caused precipitation in

CORRECTION In Vol. 60, No. 1, July 1, 1934, page 96, fourth line from the bottom, for In the presence of protective agents read In the absence of protective agents.

reacting with the specific carbohydrate, we at first regarded the A substance as a second and distinct antigenic entity, probably belonging to the group of partial antigens or haptenes. However, the analysis of Pappenheimer and Enders (3) on the chemical composition of the substance obtained by preserving an acid reaction during all steps in the purification of the specific carbohydrate, indicated a close relationship between the A substance and the specific carbohydrate. authors found that the elementary analysis of the product derived under the constant condition of a low pH was practically identical with that found for the specific carbohydrate isolated according to the procedure of Heidelberger, Goebel and Avery, during which the material is exposed to the alkaline action of concentrated barium hydroxide. Moreover, no significant difference was noted in the

<sup>\*</sup> Dr. Wu collaborated in this work during his tenure of a Rockefeller Travel-

capacity of the two substances to change the plane of polarized light. The carbohydrate obtained under the condition of acidity differed, nevertheless, in two respects from the specific carbohydrate: in aqueous solution it failed to precipitate at pH 3.4, the isoelectric point of the specific carbohydrate; it reacted by precipitating with antiserum after absorption with the specific carbohydrate. Moreover, the capacity to cause precipitation in such an antiserum was removed by boiling in dilute alkali. It was observed that the material thus treated with alkali precipitated at pH 3.4 and was able to react to titre with antiserum which contained antibody for the specific carbohydrate. Absorption of a specific antiserum with the A carbohydrate removed all antibodies precipitating either with itself or the specific carbo-These facts led to the suggestion by Pappenheimer and Enders that the specific carbohydrate of Heidelberger, Goebel and Avery represented a hydrolytic product of the A substance. They believed that possibly the A substance more closely approximated the specific antigen of Type I pneumococcus as it exists in the living cell.

During the course of the experiments which are recorded in this paper, Avery and Goebel (4) reported that by maintaining an acid reaction during the preparation, they obtained a specific substance from Type I pneumococcus, the precipitating power of which, in the presence of an antiserum absorbed with specific carbohydrate, was removed by treatment with alkali. On elementary analysis, the material appeared to be identical with the specific carbohydrate. addition they made the significant observation that this carbohydrate contained the acetyl group which was not found in the specific carbohydrate and which was apparently split off upon treatment with alkali. In their study of the immunological properties of this material, these authors showed that injection of small quantities of the acetyl polysaccharide actively immunized mice against an otherwise fatal dose of virulent pneumococci, while absorption of an antiserum with this carbohydrate appeared to eliminate its protective action in those animals—a result not obtained with comparable amounts of the substance after treatment with alkali. In the present paper a study of certain immunological aspects of the A substance purified according to the procedure described by Pappenheimer and Enders (3) is presented.

In particular the action of the substance was investigated in phagocytic systems containing normal opsonins, in bactericidal systems of leucocytes and normal and immune sera. Its capacity to absorb the mouse-protective immune body from antiserum as well as its ability to induce active immunity in mice, was also examined. The quantities necessary to induce such immunity and the time of its appearance and defervescence have been subjected to study. The sera of mice actively immunized with the material has been tested for specific antibody and the effect of injection of the A substance in A-immunized mice on their resistance to pneumococcus infection has been observed.

The material has been analyzed chemically to determine whether or not it contains the acetyl group. This has been found to be present.

The results serve to identify the A substance as prepared by Pappenheimer and Enders with the acetyl polysaccharide described by Avery and Goebel and, in general, to confirm and extend the observations of these authors and our own in respect to its immunological effects.

Preparation of the A Substance.—The A substance used in these experiments was prepared according to the procedure given by Pappenheimer and Enders (3). The data obtained on elementary analysis and determination of specific rotation have been presented in their report. Subsequent to the appearance of the paper of Avery and Goebel on the acetyl polysaccharide, this material has been subjected to analysis for the presence of the acetyl group according to the method of Friedrich and Rapoport (5). It has been found to contain 5.8 per cent acetyl. This finding, taken together with the data of the elementary analysis, appears to offer sufficient reason for concluding that the acetyl polysaccharide and the A

In the majority of experiments, the effect of the A carbohydrate was compared with the effect of the substance after boiling in alkaline solution. As Avery and Goebel (4) have shown, such treatment removes the acetyl group. Deacetylization was effected in N/50 and N/10 concentrations of alkali. After addition of the volume of either N/5 or 1 N sodium hydroxide required to give these final concentrations to a measured quantity of 1:100 A carbohydrate, the solution was immersed in boiling water for I hour and then neutralized with the required amount

Prefaration of Type I Specific Solutie Sulstance.—The specific carbohydrates designated as SSS I, SSS II and SSS III were derived from 0.1 per cent dextrose phosphate buffer broth cultures of the three types of pneumococcus, according to the procedures described by Heidelberger, Goebel and Avery.

Preparation of Antisera.—Three Type I antipneumococcus rabbit sera were used. Of these one was prepared by repeated intravenous injection of pneumococcus suspension in 0.3 per cent formolized saline. Antigens employed in the production of the remaining two were suspensions of pneumococcus killed by heating at 56° and 60°C. The route of administration was intravenous.

Strains of Pneumococci.—The strain of Type I pneumococcus used in all the experiments was of such virulence that 0.00000005 cc. of a 6 hour 0.1 per cent dextrose infusion broth culture was uniformly fatal for white mice within 48 hours. Virulence was maintained by weekly passage through mice. In a few cases in which control of type specificity was required, stock strains of Type II and III pneumococcus were utilized. The virulence of these for mice was of the same order as that of the Type I pneumococcus.

Micc.—White mice varying in weight from 15 to 20 gm. were used.

## Technique

Technique Employed in Phagocytic Experiments.—Ward and Enders (6) have described in detail this technique.

Technique Employed in Bactericidal Experiments.—In the experiments in which human defibrinated blood was used as a base for testing the antibactericidal action of the A carbohydrate against the normal antibody of the serum, the technique described by Ward (7) was followed, except that 0.25 cc. of defibrinated blood was used instead of 0.5 cc. For the study of the effect of the substance on the bactericidal power of immune antibody, a modification of the technique introduced by Robertson and Sia (8) was developed, in which polymorphonuclear leucocytes of the rabbit obtained by intrapleural injection of aleuronat, are added to the defibrinated blood of the same animal. A normal rabbit of about 2 kilos weight is injected into the right pleural cavity with 5 cc. of aleuronat suspension, prepared according to the direction of Robertson and Sia. After about 24 hours, 5 cc. of 1 per cent citrate in saline is injected into the same pleural space and withdrawn in the same syringe, together with the exudate of leucocytic cells. The suspension of cells is then slowly centrifuged for 7 minutes and then washed twice with about 20 cc. of physiological salt solution. A count of the total white blood cells present is made after resuspension in the second volume of saline, before centrifuging. A quantity of this suspension is removed which, when mixed with the desired amount of rabbit's defibrinated blood, will give a count of 10,000 per c. mm. This measured volume of suspension is then slowly centrifuged, the supernatant removed and the predetermined quantity of defibrinated blood added to the deposit of cells and well mixed with them. The number of leucocytes in the bloodleucocyte mixture is then checked. It is usually greater than that found for the saline suspension by 1,000 to 2,000 cells per c. mm. due to the presence of the leucocytes in the defibrinated blood. For satisfactory results, the final white blood count of the mixture should not be below 10,000 per c. mm. Counts from 15,000 to 17,000 do not appear to influence significantly the effectiveness of the

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A. Passive Immunization with Antipneumococcus Rabbit Serum.—Equal volumes of 6 hour 0.1 per cent dextrose infusion broth culture, diluted 1:20 in broth, and the various dilutions of antiserum are well mixed and injected intraperitoneally into mice in a total volume of 0.4 cc. Three animals were usually employed for each dilution of the serum tested. Mice serving as controls for virulence received 0.5 cc. of the culture, diluted to 1:10,000,000 and 1:10,000,000 in infusion broth. 0.1 cc. of these dilutions was plated to indicate the number of pneumococci injected. The animals were considered to have been protected if they sur-

B. Active Immunization with the A Carbohydrate.—The A carbohydrate and its derivative after treatment with alkali, were diluted in saline. 0.5 cc. of the saline dilutions was injected intraperitoneally in mice. After varying intervals the presence of active immunity was determined by injecting 0.5 cc. of 6 hour 0.1 per cent dextrose infusion broth culture of pneumococcus, suitably diluted in sterile broth. The animals were observed for a period of 8 days after injection.

Technique Employed in Absorbing Antisera with the A Carbohydrate, Its Deacetylated Derivative and with Pneumococcus Boiled on the Acid and Alkaline Sides of Neutrality.—Antibody precipitation with the A carbohydrate, or its derivative after boiling in alkali, was removed from Pneumococcus Type I antiserum by repeated addition of these antigens and removal of the resulting precipitate. Preliminary titrations were done to determine the quantity of the carbohydrate which would remove the maximum amount of antibody from a definite volume of antiscrum. This is most conveniently determined by placing 2 drops of antiscrum in each of a series of small tubes used for precipitin ring tests. One drop of the carbohydrate in serial dilutions, ranging from 1:200 to 1:1,000, is added to each tube. The contents of the tubes are well mixed and allowed to stand at room temperature for 2 hours. The precipitates are thrown down in the centrifuse and on the supernatant fluid is layered the carbohydrate, diluted 1:2,000.

The original mixture of highest dilution of carbohydrate and antiserum which fails to give a Positive ring test is taken to indicate optimum proportion of antigen and antiscrum. On this basis, the amount of carbohydrate required to remove the antibody from any desired volume of antiserum is calculated and added in

the smallest possible volume of diluent. After standing at 37°C, for 1 hour and overnight in the ice box, the precipitate is removed by centrifuging and the supernatant tested with the carbohydrate diluted 1:200 and 1:2,000. If any precipitate is observed the absorption is repeated according to the procedure described above.

18 hour 0.1 per cent dextrose infusion broth cultures of Type I pneumococcus were centrifuged and the deposit resuspended in a volume of saline equivalent to that of the broth removed. The saline suspension was divided into two equal portions. To one, sufficient acetic acid was added to give a final concentration of N/100. To the other, sodium hydroxide was added to give the same concentration of alkali. The suspensions were then allowed to stand in the Arnold sterilizer for an hour at 100°C. After neutralizing they were centrifuged. Absorption of Pneumococcus Type I antiserum, diluted 1:4, was twice carried out with the deposit from 100 cc. of the saline suspension. The mixture of serum and organisms stood for 1 hour at 37°C. following each absorption.

### EXPERIMENTAL

# A. The Effect of the A Carbohydrate on the Phagocytosis of Type I Preumococcus in Normal Human Blood

In this experiment, the results of which are presented in Table I, the effect of the A carbohydrate and its derivatives on the phagocytic power of normal human defibrinated blood was determined. It was found that the A carbohydrate in a concentration of 1:2,000 practically eliminated the opsonic capacity of the blood, whereas the substance after boiling in N/10 and N/50 sodium hydroxide, or the soluble specific substance (SSS I) prepared according to the procedure of Heidelberger and his associates in the same concentration, reduced the count only about two-thirds. It will also be noted that, in the case of the A carbohydrate, there is a definite reduction in the count in a concentration of 1:512,000 while the action of its derivatives is not definite in concentrations above 1:8,000. By this method of comparative testing, it appears that the A carbohydrate is at least 64 times as effective in removing opsonins from normal serum as the deacetylated polysaccharides. The effect of the A carbohydrate is type-specific since it has no action on the opsonic titre of the same blood in the presence of Type II pneumococcus. These results possess an additional interest in that they elucidate the finding of Ward and Enders (6) concerning the failure of SSS I to remove completely the phagocytic power of normal human blood against Pneumococcus

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T		TABLE I		
Type of pneumococcus				
	Carbohydrate	Conn		
		Concentration of carbohydrate*	No. of organisms	
I		Jurate	phagocyted be	P
1	0		phagocyted by 50 cells	Percentage of cells
	ı	0		taking part
1	A		279	
1	- 1	1:2,000	1 1	98
- 1	- 1	1:8,000 l	12	
1	1	1:32,000	52	20
	1	1:128.000	85	46
1	1	4:312 nnn   1	63	62
1	1	1:2,048,000	120	58
A	boiled N/50	/10,000	259	76
1.	NaOHt	:2,000	~9	96
1	1	:8,000	112	20
	1	32,000		0.4
1	1 1:	128,000	169	84
1	1:	513.00	290	90
	1 1	512,000	258	100
1 .	300 -	2,048,000	302	98
	SSS I 1:2	00-	295	100
1	1 1:0	,000	. 1	100
	1.70	000	132	
1	1.32	,000	182	90
1	1:12	8,000	218	90
	1 1:517	2000 1	223	92
II	1:2,0	48,000	306	96
/ 0	) [	2	198   198	98
1	(	)	10	o o
A	1.	61	16	
Concentration	1:2,000	o /	100	)
Concentration of cari Titration of A boiled those for A boiled in	bohydrate	/ 79	0	•
those for A boiled	in Nice Present	t in the	100	
A boiled in	10 NaOH	rare phagocr	tic con	
	"NOON NAOH	oure counter.	system	

<sup>†</sup> Titration of A boiled in N/10 NaOH gave counts not significantly different from those for A boiled in N/50 NaOH.

# B. The Effect of the A Carboliydrate on the Opsonin in Anti-Type I

Anti-Type I pneumococcus serum, absorbed with the A carbohydrate and with that substance boiled in N/50 sodium hydroxide, was added in suitable dilutions to normal defibrinated human blood which,

by itself, exhibited only a feeble phagocytic action against Type I pneumococcus. The results are summarized in Table II. same experiment these absorbed sera were titrated for opsonic power in a system containing the exudative leucocytes and defibrinated blood of a normal rabbit prepared according to the method described for preparing this system for use in bactericidal tests. The results were entirely comparable to those recorded in Table II. From the data recorded therein, it was calculated that the immune opsonin was reduced in the serum absorbed with A carbohydrate to about 1.6 per cent of its original titre. The deacetylated polysaccharide was less effective in removing the opsonin of the serum, since after absorption from 6.3 to 26 per cent of the antibody remained. This difference in the percentages of residual antibody after absorption with deacetylated polysaccharide depends upon whether the counts denoting the endpoint of the action of the immune serum are taken as a basis for calculation or those which indicate no diminution in the maximum opsonic effect, as compared with the count obtained with the unabsorbed antiserum. It is necessary to point out here that the antibody, although greatly reduced by absorption with the A carbohydrate, was not completely removed. This fact, we believe, need not be explained by assuming the presence in the serum of any qualitatively distinct antibody, but most probably may be attributed to the conditions upon which the reaction between antibody and the antigen in solution depend. In favor of this view is the fact that suspensions of pneumococcus, boiled in N/100 acetic acid, have been found capable of removing even more opsonic antibody than the A carbohydrate. That this substance is the antigen involved, but obviously in a somewhat different physical state, is indicated by the fact that absorption of the serum with organisms boiled in N/100 sodium hydroxide only slightly reduces its opsonic titre. Such absorbed serum fails to precipitate with the deacetylated polysaccharide, but reacts to titre with the A carbohydrate.

On the basis of these experiments, it is apparent that the A carbohydrate is decidedly more effective in reducing both the normal and immune opsonin involved in the phagocytosis of Type I pneumococcus.

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	TA	BLE II		. 1
	Anti-Type I pneumococcus serum	Dilatio	132	
	0	antiserum	No. organ phagocyt by 50 ce	Percentage of cells taking part
	Unabsorbed	1:256	66	50
\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\	- 1	1:1,02 <u>4</u> 1:4,006	502 396	100
Normal human defibri- nated blood + sus- pension of Tra-	psorbed with A	1:16,384 1:1	162 100	100 96 74
pension of Type I  pneumococcus		1:4 1:16	460 482	96
		1:64 1:256	333 161	100 · 98
	rbed with A led in N/50	:4 :16	506	88 62
	$\begin{array}{c c} & & 1: \\ & & 1: \end{array}$	256 Z	770 524	100 100
The Effect of the	1 1.	• - 1	200	100 94 .
The Effect of the A Carbon	hydrate on the	Backericia	69	76 60

# C. The Effect of the A Carbohydrate on the Bactericidal Action of Normal

Employing the defibrinated blood of the same normal individual which formed the base for the opsonic experiment, the antibactericidal action of the A carbohydrate was compared with that of SSS I, pre-Pared according to the method of Heidelberger, Goebel and Avery. The results recorded in Table III parallel those obtained using the opsonic technique. It is evident that the bactericidal action of blood is significantly depressed by as small a concentration of the A carbohydrate as 1:512,000. The concentration of SSS I required to produce the same inhibition of bactericidal capacity is 1:32,000 which represents at least a sixteenfold decrease in activity. It will be noted that the A carbohydrate does not significantly influence the capacity of the same blood to kill Type II pneumococcus. Essentially similar findings have been recorded in a comparison of the antibactericidal effect of the A carbohydrate and that substance after boiling in N 10 sodium hydroxide.

TABLE III

Type of pneumococcus	Carbohydrate	Concentration of carbohydrate	Approximate No. pneumococci killed by 0.25 cc. blood	
I	0	_	40,000	
	SSS I	1:2,000 1:8,000 1:32,000	40 400 400	
	Α	1:128,000 1:2,000 1:8,000	40,000 0 4	
		1:32,000 1:128,000 1:512,000	4 40 400	
11	0 A	1:250	400,000 40,000	

## D. The Effect of the A Carbohydrate on the Bactericidal Action of Anti-Type I Pneumococcus Rabbit Serum

The determination of the effect of the A carbohydrate on the bactericidal action of anti-Type I pneumococcus rabbit serum was carried out in two different ways. In one, a suitable dilution of the antiserum was chosen and held constant while the quantity of A carbohydrate or its deacetylated derivative was varied. In the other type of experiment, the antiserum was absorbed with the A carbohydrate or its derivative and serial dilutions of these absorbed sera were tested for bactericidal power. In both cases a mixture of exudative leucocytes and defibrinated blood of the normal rabbit was used as the basis of Both methods of testing showed that the A carbohydrate was several times more efficient in impairing the ability of the antiserum to promote the destruction of the pneumococcus in the presence of fresh normal serum and polymorphonuclear leucocytes. It is, however, to be emphasized that by the method of absorption it was found that in every case a not inconsiderable amount of the antibody involved in the destruction of the organism remained. failure to absorb completely all antibody active in the bactericidal

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# TABLE IV

# Effect of Carbohydrates on Constant Dilution of Anti-Type I Pneumococcus Rabbit

	Constant D	ilution	
Concentration of antiserum		Serum Of Anti-Ty1	be I Pneumococcus Rabi
	Carbohydrate	Concer	Nabi
1:2,000	0	carbohydrate	Approximate No.
2,000	0	_	O Zimed
1	A	_	70,000
	- 1	1:2,700 1:8,000	
1	1	1:16,000 1:32,000	0 7
1.		1:64,000 1:128,000	700 700
A	boiled in x/50	1:2,700	7,000 70,000
		1:8,000	7.00a
Baclericidal Action of	Experiment Anti-Type I	II	70,000
	- JPE I Pman		

# Bactericidal Action of Anti-Type I Pneumococcus Rabbit Serum Absorbed

Antiserum absorbed with carbohydrate	with Carbohydrates	us Rabbit Serum Absorbed
absorbed with carbohydrate	Concentration of antiserum	
Unabsorbed	_	Approximate No. pneumococci
,	1:8,000 1:32,000 1:128,000	7,000
A	1:32	700 0
A boiled in N/50 NaOH	1:128 1:500 1:2,000	70,000 700
NaOH	1:500	70 0
	1:2,000 1:8,000 1:32,000	70,0 <sub>00</sub> 70
	55,000	, , , , , , , , , , , , , , , , , , ,

system was expected from the results obtained when similarly absorbed serum was tested for residual opsonin and, we are inclined to believe, is due to the same cause that we have previously postulated. In discussing these experiments, it is important to note the fact that it proved impossible—using the constant dilutions of antiserum 1:250 and 1:500-to show any significant effect of the deacetylated carbohydrate in the highest concentration used (i.e., 1:2,700) on the bactericidal property of these quantities of antiserum which, however, were definitely impaired by dilution of the A carbohydrate in concentrations ranging from 1:16,000 and 1:32,000. In Table IV the results of two experiments among several carried out are summarized. port the observations already described in showing that the A carbohydrate is about sixteenfold more active in removing antibody than is the deacetylated polysaccharide. Nevertheless, it requires approximately equal concentrations of antiserum and A carbohydrate before complete inhibition of the bactericidal action is obtained. The type specificity of the action of the highest concentration of A carbohydrate was assured by adding it to Type III pneumococcus, together with diluted anti-Type III pneumococcus serum. It exerted no antibactericidal effect in this system.

## E. The Effect on the Mouse-Protective Action of Anti-Type I Pncumococcus Rabbit Serum Absorbed with the A Carbohydrate

Although repeated efforts were made to show that the A carbohydrate would remove all protective action for mice from anti-Type I pneumococcus rabbit serum, none were completely successful. From the data recorded in Table V, it is clear that absorption with the A carbohydrate markedly reduces the protective titre of the antiserum. It is difficult, because of the small number of animals involved, to assess precisely the extent of this reduction, but if one selects the highest dilution of serum which protects three out of three mice as a basis for calculation, it will be found that absorption with A has lowered the titre to 1.5 per cent of its original value, compared with a reduction to 6.2 per cent after absorption with the deacetylated compound. These figures indicate that the A carbohydrate is about four times more effective in removing the protective titre of the antiserum. Experiments of the same type in which the A carbohydrate used for

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absorption was boiled in N/10 NaOH yield comparable results. the protection test carried out with serum absorbed with A and its derivatives shows less difference in activity between them than is 139 revealed either by the bactericidal or opsonic techniques. a greater discrepancy in absorptive capacity is found if portions of the serum previously saturated with Type I pneumococcus boiled in N/100 However,

Effect on the Mouse-Protective Titre of Anti-Type I Pneumococcus Rabbit Serum

Absort	Tilre of	Anti T		
Dilution of antiserum	d with the	3'pe	I $P_{neum}$	ococcus Rabbit Serum
		Carbo	ohydrate.	Rabbit Serve
Unabsorbed serum	1:4	1:16		
· o din	No.		1:64	The state of the s
1	Not done	Not done	,,,	1:256 1:1,024
Sam	- 1	Not done	Not done	s
Serum absorbed with A	- 1	- 1	- 1	
THE A	. 1	- 1	- 1	$\begin{array}{ccc} S & \int & D \\ S & \int & D \end{array}$
1	9	s	_	"   D
Serum absorbed with A boiled	S S S	s	S D	$D = \int_{X_{-1}}$
in N/50 NaOH with A boiled	·	D	$\mathbf{D} \mid$	D Not done
NaOH 1 boiled	s l	- 1	<i>D</i>	D
1	s s	s s	s	1
Serum absorbed with Pneumo-	s	S	SI	S
coccus I boiled in N/100 HAc	- 1		ח ו	$\begin{array}{c c} D & D \\ D & D \end{array}$
ooned in N/100 HAC	s   s	s	1 :	$D \setminus D$
s ( )			) h.	_
Serum absorbed miss			) /-\ot	done Not done
Scrum absorbed with Pneumo-	1 ~	D	1	- 40116
- HOP VaOH	s	- 1	- 1	1
Three mi	S	S	1 5	1
D = died received the	S	S	l s	S
D = died. S = survived.	0n of		S S S	D
Three mice received the same diluti  D = died. S = survived.  acetic acid and x/100	or anti	serum.		D
tite acid and v/100				

acetic acid and N/100 sodium hydroxide are tested for their protective titre in mice. The results of an experiment of this kind are included in Table V. It will be seen that absorption with pneumococci boiled in alkali does not definitely affect the protective action of the serum, although this treatment had removed all precipitating antibody reacting with the deacetylated carbohydrate. In contrast, absorption with organisms boiled in N/100 acetic acid removes a large portion of the

protective action. There can be little doubt that it is the A carbohydrate present in the pneumococcus upon which the removal of the antibody depends.

This failure to eliminate completely the mouse-protective action of an antiserum by addition of the A carbohydrate is not in entire agreement with the findings of Avery and Goebel (4). In addition to the possibility which has already been discussed concerning the physical state of the antigen, the discrepancy may be either in the fact that these authors did not test serum less dilute than 1:10, or that they employed antipneumococcus horse serum, while we used antipneumococcus rabbit serum. Powell and his coworkers (9) have shown that heterophile antibody of Forssman, together with very small quantities of specific pneumococcus antibody, greatly increases the therapeutic properties of the serum in rabbits. Since the Forssman antibody may be present in antipneumococcus rabbit serum, it is possible that its presence in the antiserum employed might account for this difference in results.

## F. The Antigenic Action of the A Carbohydrate

It has been found that when comparatively large amounts of the A carbohydrate are injected into mice no immunity against virulent Type I pneumococcus develops. If the quantity of carbohydrate is decreased, definite, though limited resistance to infection develops. Boiling the A carbohydrate in N/10 sodium hydroxide removes completely its capacity to function as a complete antigen. Similar treatment in N/50 sodium hydroxide appears to only partially destroy the antigenic property of the substance. Briefly summarized, the experimental findings were as follows: Nine mice were vaccinated by three intraperitoneal injections of 0.00025 mg. of the A carbohydrate, administered every 3rd day, and infected with 0.5 cc. doses of Pneumococcus Type I, increasing by tenfold from 1:1,000,000 to 1:10,000 dilution of broth culture, on the 6th day after the last dose of the carbohydrate. Two only, of the nine mice, died with pneumococci in the heart's blood. These animals had received the largest number Out of four mice vaccinated in the same manner, with of organisms. equivalent quantities of A boiled in N/50 sodium hydroxide, two survived. Of eight animals vaccinated with A boiled in N/10 sodium

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JOHN F. ENDERS AND CHAO-JEN WU

hydroxide and infected with doses of pneumococci ranging from 141 Quantities of A as large as 8 mg. given at 3 day intervals, in doses of

2 mg. each, failed to confer any protection on the 11th day after the last injection. It was thought that if a longer period was allowed to elapse, subsequent to the course of immunization with such large doses, before the animals were infected, immunity might develop. Accordingly, an interval of 6 weeks was allowed before infecting two mice, one of which had received 1 mg. and the other 2 mg. of the A carbohydrate. No indication of resistance was observed. Additional confirmation of the fact that large doses of the A carbohydrate fail to immunize is given in Table IV. It was believed unnecessary to present these experiments in greater

detail, since they fully confirm those of Avery and Goebel (4). We have noted that extremely small quantities of the A carbo-

hydrate may actively immunize mice: 0.5 cc. of 1:10,000,000 dilution of the material (i.e., 0.00005 mg.), administered intraperitoneally, at a single dose, will protect a certain percentage of the animals under test, against at least 10 M. L. D.'s of virulent pneumococci. Increasing the immunizing dose tenfold appears to improve slightly the immunity obtained. This dose of 0.0005 mg. proved to be about the optimum, since a further increase to 0.005 mg. is definitely less effective. Larger amounts of the A carbohydrate produce practically no resistance to infection. There is a suggestion that with a dose of 0.005 mg. some immunity may develop within 24 hours. After 3 days 50 per cent of the mice vaccinated with 0.00005 and 0.0005 mg. showed resistance. Maximum immunity exists from 6 to 25 days after vaccination, but apparently is in a state of defervescence by the 49th day. These statements are based on the experimental results included in Table VI. The manner of procedure was as follows: 60 normal mice were divided into five lots. The twelve mice in each lot were injected intraperitoneally with the same dose of A carbohydrate. The quantities received by the various lots of mice ranged from 0.00005 mg. to 0.5 mg. At varying intervals after vaccination, two mice were selected from each lot and injected intraperitoneally with the same dose of Type I pneumococci, which was equivalent to at least 10 M. L. D. In every case the heart's blood of the mice which died was cultured.

TABLE VI

	1								
Lot No. of	Amount of A	Two	Two mice from each lot injected intraperitoneally with 0.5 cc. culture Pneumococcus I diluted 1:1,000,000						
mice	used for vaccination		Days after va	accination on whic	h mice we	ere infected			
		1	3	6	12	25	49		
1	mg. 0.00005	D 24 D 48	D 40 S	D 98 HB-	D 60 S	S S	D 36 D 60		
2	0.0005	D 48 S	D 40 D 70 HB-	D 48	S S	D 144 S	D 36		
3	0.005	D 48 D 60	D 40 D 48	D 48 S	D 60	D 60	D 36		
4	0.05	D 48 D 48	D 40 D 48	D 48 D 60	D 36 D 36	S D 36	D 36 D 36		
5	0.5	D 24 D 48	D 40 D 36	D 24 D 48	D 36 D 36	D 36 D 36	D 44 D 44		
Normal mice injected intraperitoneally with 0.5 cc. 1:10,000,000 culture Pneumococcus I		D 48	D 36	D 48	D 36	D 60	D 60		

D= died; numerals following indicate number of hours after infection in which death occurred. S= survived. HB-= heart's blood culture negative. In all cases not indicated, culture of heart's blood was positive for pneumococcus.

# G. Elimination of Immunity Induced by Vaccination with the A Carbohydrate

It became of interest to ascertain whether the immunity resulting from vaccination with the A carbohydrate could be annulled by injecting that substance just prior to infection with the pneumococcus. Five mice were given three injections of 0.00025 mg. intraperitoneally, every 3rd day. 9 days after the last injection, three of the mice were injected intraperitoneally with 0.5 mg. of the A carbohydrate. After an interval of  $4\frac{1}{2}$  hours, these animals, together with the two which had been vaccinated but had received no immediate preliminary

within 3 days, together with the normal control. Of the two vaccinated mice which had not received 0.5 mg. of A before infection, one survived 6 days and the other was killed after 7 days. Preliminary

injection, then, of a comparatively large quantity of the A substance

shortly before infection appears to remove the protective mechanism developed in response to small doses of the same material previously

H. Passive Transfer of Immunity Developed in Response to Injection

The question arose as to whether demonstrable antibody appeared in response to vaccination with the A carbohydrate in the serum of the mouse. Eight mice were immunized at the same time, and according to the procedure in the preparation of animals used in the previous experiment. 10 days after the last immunizing dose of the A carbohydrate was given, the mice were bled from the heart under ether anesthesia, employing a tuberculin syringe and a 27 gauge hypodermic needle. The blood was pooled and allowed to clot. About 4 cc. of serum were obtained. Four normal mice were each injected intravenously with 0.75 cc. of this pooled serum. At the same time, two normal mice were injected, by the same route, with 0.75 cc. of the Pooled serum of four normal mice. After an interval of 20 hours, these six animals were infected intraperitoneally with 0.5 cc. of Type I Pneumococcus culture, diluted 1:1,000,000. A normal mouse was injected with 0.5 cc. of 1:10,000,000 dilution of the same culture. The results which are presented in Table VII show clearly that the serum of mice immunized with the A carbohydrate contains substances

protective against Type I pneumococcus.

With the remainder of the pooled serum obtained from the mice vaccinated with the A carbohydrate, tests for the presence of agglutinins for Pneumococcus Type I and precipitins for the A carbohydrate were carried out. In both cases the results, entirely negative,

indicated that the amount of antibody present was very small.

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TABLE VII	•
Passive Transfer of Protective Substance in Serum of Mice Vaccinated with	the A
Carbohydrate	

Mouse	Pooled serum A- vaccinated mice	Pooled serum nor- mal mice	Dilution of infecting dose Pneumococcus I (0.5 cc.)	Result
	cc.	cc.		
1	0.75	0	1:10,000,000	S
2	0.75	0	1:1,000,000	S
3	0.75	) o	1:1,000,000	S
4	0.75	0	1:1,000,000	S
5	0	0.75	1:1,000,000	D
6	0	0.75	1:1,000,000	D
7	0	0	1:10,000,000	D

Serum administered intravenously, pneumococcus culture intraperitoneally 20 hours after serum was given.

### DISCUSSION

In general the experimental evidence presented shows that the A carbohydrate, which, on the basis of chemical analysis and biological action, is to be identified with the acetyl polysaccharide of Avery and Goebel, is distinctly more effective than the deacetylated derivative in reducing the efficiency of the several immunological systems which have been studied. The A carbohydrate, then, closely approaches, if indeed it be not identical with the substance developed by Type I pneumococcus which enables the organisms to resist the defensive mechanisms of the host, in so far as these are type-specific.

Capable, like the organism itself, of inducing resistance to subsequent infection, as Avery and Goebel have shown for the acetyl polysaccharide, the A carbohydrate, introduced parenterally into mice, arouses a state of active immunity which may appear as early as the 3rd day after vaccination. This immunity, closely correlated with the quantity of carbohydrate employed—since doses of over 0.005 mg. fail to protect—is not of great magnitude nor of marked duration. It may be completely eliminated by introducing into the body of the vaccinated mouse an appropriate amount of A carbohydrate shortly before infection. That such resistance is dependent upon substances which may appear in the serum has been demonstrated by the passive transfer of the immune state to normal mice from animals vaccinated with the A carbohydrate.

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Perhaps the chief interest and value of these experiments with the A carbohydrate and those of Goebel and Avery with the acetyl polysaccharide lie in their application to our ideas on the nature of antigens. 145 It is extremely improbable that the acetylated carbohydrate contains even a minute quantity of protein, yet it functions as a complete antigen, in that it not only actively immunizes the animal body against subsequent invasion by the homologous organism, but also gives rise to demonstrable protective substances in the serum. Tentatively, it would seem that the conception of all complete antigens as being protein in nature should be modified.

There is a question which has been in our minds during the course of this work and which still remains unanswered: Is there one antibody which reacts in a quantitatively different manner with the A carbohydrate and the deacetylated compound, or are there two antibodies corresponding to the two forms of the antigen? Against the acceptance of the first alternative is the fact that a serum containing a high titre of precipitin reacting with the A carbohydrate may fail entirely to precipitate with the deacetylated material. On the other hand, there is ample evidence to show that the introduction into an antigen of chemical groupings, no more complex than the acetyl group, may definitely change the specificity. However, if two antibodies are postulated, it would be necessary, in view of experimental fact, to assume that the A carbohydrate could unite with both, whereas the deacetylated carbohydrate could react with only that antibody specifically produced in response to its antigenic stimulus.

In this connection, the experiments of Avery, Goebel and Babers (10) on the serology of artificially prepared antigens containing substituted glucosides present an interesting analogy to the behavior of the A carbohydrate and the deacety-lated substance. These authors showed that an antiserum prepared by injecting p-aminophenol a-glucoside linked to globulin was precipitated by both p-aminophenol  $\alpha$ -glucoside-globulin and  $\beta$ -aminophenol  $\beta$ -glucoside-globulin. If p-aminophenol α-glucoside was added to this antiserum, precipitation was inhibited in the presence of both a- and \(\beta\)-glucosides linked to globulin. However, addition of β-glucoside inhibited precipitation only against p-aminophenol p-glucoside linked to globulin. Since in this case apparently only one antibody is concerned, their findings

indicate that the difference in reaction capacity of the A carbohydrate and the deacetylated product could be explained on the assumption that only a single antibody was involved.

### SUMMARY

- 1. The A carbohydrate isolated from Type I pneumococcus by Pappenheimer and Enders, on the basis of elementary analysis, the presence of the acetyl group and its immunological properties, appears to be identical with the acetyl polysaccharide described by Avery and Goebel.
- 2. The A carbohydrate possesses a greater anti-opsonic action than either the deacetylated substance obtained by boiling in alkali or the soluble specific substance of Type I pneumococcus prepared according to the procedure of Heidelberger, Goebel and Avery. The opsonic titre of normal human serum is practically eliminated upon the addition of the A carbohydrate—an effect not observed with equivalent amounts of either the deacetylated material or the specific soluble substance. In immune serum, the A carbohydrate brings about a quantitatively greater reduction in opsonic activity than its derivatives, but it has not been possible to demonstrate complete inhibition of phagocytic action by the method of absorption of antibody.
- 3. In a system of normal human serum and leucocytes capable of destroying Type I pneumococcus, the bactericidal effect may be entirely removed upon the addition of the A carbohydrate. It proved impossible to inactivate the bactericidal action with the deacetylated substance in equivalent proportions. In this system, the A carbohydrate was about 64 times more effective as an antibactericidal agent than the deacetylated compound. Essentially similar results were obtained in a study of the antibactericidal properties of the A carbohydrate and the deacetylated derivative in the presence of anti-Type I pneumococcus rabbit serum added to a mixture of exudative leucocytes and the defibrinated blood of the rabbit.
- 4. The mouse-protective titre of anti-Type I pneumococcus rabbit serum is lowered to a greater degree after absorption with the A carbohydrate than it is by similar treatment with the deacetylated compound. Absorption with the A carbohydrate does not, however, completely remove the protective antibody.

5. As Avery and Goebel have shown in the case of the acetyl polysaccharide, so the A carbohydrate, when administered in very small quantities, protects mice against an otherwise fatal dose of Type I pneumococcus. Active immunity in mice has been obtained with as little as 0.00005 mg. of the A carbohydrate administered in a single dose. Doses larger than 0.005 mg. confer no protection on these 7 animals. Deacetylization of the A carbohydrate after boiling in N/10 : 1:3: 2: sodium hydroxide destroys its protective capacity while similar treat-ment in N/50 alkali does not completely remove its immunizing prop-erty. Active immunity may arise within 3 days following a single injection of the A substance. It appears to be at its height from 6

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- to 25 days thereafter, and is retrogressive by the 49th day following vaccination. Injection of the A carbohydrate into immunized mice immediately before infection deletes the state of resistance. 6. The immunity actively induced as a result of injection of the A carbohydrate may be passively transferred to normal mice with the serum of vaccinated animals.
- 7. Since the evidence obtained in the course of this study indicates that the A carbohydrate of Type I pneumococcus and the acetyl polysaccharide of Avery and Goebel represent the same chemical substance, it is suggested that the designation "A carbohydrate" or "A substance" be relinquished in favor of the more exactly descriptive term "acetyl polysaccharide." BIBLIOGRAPHY
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# VARYING INFLUENCE OF TUBERCULOUS RABBIT PLASMA ON THE GROWTH OF FIBROBLASTS IN VITRO

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In studying tuberculin reactions by means of tissue cultures attempts were made to "sensitize" normal fibroblasts in vitro. It soon appeared that plasma from rabbits with early tuberculosis had a different primary influence from that obtained later in the disease; hence these differences were more carefully investigated. This report deals with the results of that investigation.

Animals.—In an attempt to eliminate the variables due to differences of age and sex, litters of known age were obtained from a stock of rabbits bred in the Institute. The animals were so selected that a tuberculous and a non-tuberculous litter mate of the same sex were kept as pairs; the tuberculous animals were housed in a different room from the controls, but were given a similar diet. The type of rabbit used—hybrid of English, lilac and Havana—has been subsequently shown to be relatively highly resistant to tuberculosis; hence the disease probably ran a more chronic course than usual. At the time of inoculation the animals were immature and varied from 1,500 gm. to 1,800 gm. in weight. The majority of them lived from 5 to 10 months after inoculation, some over a year; they succumbed finally to the chronic form of the disease, often after months of testicular, arthritic or ocular tuberculosis.

Inaculum.—The rabbits were inoculated intravenously with 0.1 mg. of an 11 to 13 day culture of a bovine strain of tubercle bacilli, B 1, grown on Petrofi's gentian violet egg medium. It has subsequently been shown that these cultures probably contained a mixture of smooth and rough forms. The growth was harvested from the surface, weighed wet, then ground in a mortar with Ringer's solution until a uniform suspension resulted. The coarser granules were removed by slow centrifugalization for 3 minutes. The subsequent suspension was suitably diluted so that 0.5 cc. contained 0.1 mg. of bacilli. This amount was injected into the marginal car vein; and in all but one animal these veins remained free of

Tissue Cultures.—The cells used for testing the influence of the plasma were so called fibroblasts grown from the testes of adolescent rabbits. Under aseptic precautions, the testes were removed from animals immediately post mortem or while under full ether anesthesia, cut into small bits, of which four pieces were explanted into a medium consisting of heparinized rabbit plasma and tissue extract. They were washed and renourished twice a week with plasma or tissue extract; after 10 to 14 days healthy appearing growths were divided into four pieces and transplanted into similar medium. At the time of an actual experiment each transplant was carefully excised from the medium in which it was growing, divided into four equal sized portions, each of which was placed in a different Carrel culture flask. Four such transplants from four different original pieces were thus placed in similarly marked segments of four different flasks. With this arrangement the subcultures from two of these stock transplants were used as controls and two as the objects for testing the influence of the abnormal environment. Under these conditions the factor of varying initial growth energy was controlled as well as possible, for by identifying each subculture with a distinctive mark each of the four could be compared with its fellows having a common source.

Plasma.—Except in one or two instances where serum was employed the fluid containing the growth-influencing substances consisted of plasma prepared as follows: Blood was allowed to drop from a small incision in the marginal ear vein of the rabbit directly into tubes containing 0.5 cc. of 0.1 per cent heparin dissolved in Ringer's solution until a total volume of 4 cc. was obtained. The tubes were placed immediately in ice, and centrifuged after all of the specimens had been collected. The supernatant plasma was pipetted into other tubes and kept cold until used in the cultures.

Tissue Extract.—Three different extracts were used, two in preliminary studies and the third in the main portion of the work. At first, chick embryonic extract was tried, but soon proved to be inferior in stimulation of rabbit fibroblasts as compared with homologous extracts; moreover, when heterologous extracts were used the effect of growth inhibiting substances was accentuated. Rabbit splenic extract was more satisfactory, but was much more costly than rabbit embryonic extract, since larger amounts of the latter could be obtained from one animal. 16 day rabbit embryos were separated from the placentae and membranes, weighed and passed through a Fischer embryonic tissue crusher, then mixed with enough Tyrode's solution to make a 25 per cent suspension. This was centrifuged and the supernatant turbid fluid used as the plasma-coagulating reagent. Unless this extract were to be employed within a few days it was preserved in a frozen state, for in this condition it appeared to retain its activity for weeks.

In most of the actual experiments the cultures were set up in two phases: solid and liquid. After the four selected transplants were placed in the four quadrants respectively of a Carrel D or micro flask containing 0.5 cc. of heparinized normal rabbit plasma, 0.25 cc. of 25 per cent tissue or embryonic extract was added; then the flasks were transferred to a warm stage at 37°C. in order to facilitate coagulation of the plasma. Thus far, the environmental conditions of the transplants

in a given experiment were common to all. The flasks were now arranged in pairs so that each pair contained transplants from a common source. Excess of the embryonic extract was removed by placing 0.5 cc. of Tyrode's solution in each flask, allowing to stand half an hour, then pipetting it off; this maneuver was repeated. Then to one of each pair of flasks was added 0.25 cc. of plasma from a tuberculous rabbit and 0.25 cc. of Tyrode's solution, and to the other similarly diluted plasma from a normal sibling of the same sex. The flasks were incubated at 37°C. and examined daily. On the 3rd or 4th day they were opened, washed with Tyrode's solution, as described above, and 0.5 cc. of fresh 50 per cent plasma from the same rabbits as previously used, was added to each. Again, they were

In the last two experiments the procedure was modified somewhat: Instead of employing a solid phase of normal plasma and a liquid phase of tuberculous or control plasma, the transplants were placed immediately in 0.7 cc. of the tuberculous or non-tuberculous plasma respectively, and 0.3 cc. of 25 per cent rabbit embryonic extract. As soon as the coagulum was solid the flasks were stoppered and placed in the incubator without being subjected to the maneuver of washing and adding fresh plasma. Subsequently, however, these cultures were washed and renourished with 50 per cent plasma as described above.

Estimation of Growth.—The cultures were examined microscopically at frequent intervals in order to compare the appearance of the cells growing under different environments. The amount of increase in growth was recorded according to the method of Ebeling (1) by means of outline drawings made with the aid of an Edinger projectoscope. The areas thus outlined were measured with a planimeter. In each experiment from 8 to 16 transplants were subjected to the influence of the abnormal plasma, with a corresponding number of controls. The total areas of all the transplants growing under any particular environment on a given day were combined and from these totals the increments of growth were estimated. In this way the errors resulting from individual variations in rates of growth and inherent in the method were reduced to a minimum. Infected cultures and those in which the growth was obviously influenced by factors other than those experimentally introduced were eliminated from the calculations. than those experimentally introduced were eliminated from the calculations. The influence of the abnormal plasma is expressed by the ratio Tuberculous plasma.

Normal plasma

when distinctly less than 1 it indicates that growth-depressing influences in the environment predominated, and, when more than 1, that growth-stimulating factors were dominant.

The results obtained in eighteen different experiments are combined and set out in detail in Table I. The increments of growth on the days indicated are shown for each experiment; the data obtained with tuberculous plasma are given first for each pair, and those ob-

Comparative Rates of Growth of Fibroblasts in Tuberculous and Normal Plasma TABLE I

		•								1	21110			
Experiment	Rabbit No.	Condition	Duration of					ı	Day	ð.				
			tunercuiosis		2	3	4	'n	0	1	80	6	2	=
T 37	3 pooled*	Tb Normal	21 days	Increment	3.7	11.0	25.4	31.0						
				Ratio $\frac{Tb}{Nl}$	1.06	1.05	0.84	0.64						
T 63	34-65 34-71	Tb Normal	18 days	Increment "	1.5	4.7	10.6	17.4	25.4		35.0			
				Ratio $\frac{Tb}{Nt}$	1.0	0.92	16.0	1.06			0.97			
T 65 a	34-67 34-71	Tb Normal	24 days	Increment "	0.52	1.5	2.6	5.1	<del></del>	······································	11.0	14.6	***************************************	25.0
				Ratio $\frac{Tb}{Nl}$	1.0	1.15	0.97	0.75		· · · · · · · · · · · · · · · · · · ·	0.55			0.52
T 65 b	34-65 34-69	Tb Normal	24 days	Increment	0.53	1.7	3.1	7.5	<del></del>		16.6	23.1		35.5 41.6
				Ratio $\frac{Tb}{Nl}$	1.2	6.0	16.0	0.94			0.77	0.81	<del></del>	0.85
T 66	34-65 34-69	Tb Normal	31 days	Increment	1.2	3.6		8.6	16.7		32.2	<u></u>		
				Ratio $\frac{Tb}{Nl}$	9.0	0.3		0.46			0.64			
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Serum was used instead of plasma.

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-	3 5.0 5 10.3 2 0.48	4.7     12.5     20.0       1.01     15.6       1.25     1.28	21.2	8.4	23.8 42.7 0.73 0.74	23.8 23.8 42.7 0.81 0.77	13.5 22.4 11.7 22.0
Increment		1.50	0.57	44 2.7	0.85 0.71	1.3	0.54   2.3   4.9   1.24   1.15   1.16
Normal 31 days	Th Normal 38 days	Th Normal 66 days	mal 73 days	6 wkg.	Ratio To NI Increment	Ratio Tb NI O MI NI	Ratio Tb
31.71	T 68 31.65	771 31.67	T 72 31-67 Tb	T 79.4 31.70 Tb 31.71 Normal	T 70 h 31.65 Tb 31.71 Normal	T 59 c 32.86 Tb 32.89 Normal	

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Experiment No	Rabbit No.	Condition	Duration of						Day	۸				
			tubercurosis		2	3	4	25	9	7	8	6	10	=
Т 88 а	36-25 36-26	Tb Normal	16 days	Increment "		3.5		4.6		8.7				
				Ratio $\frac{Tb}{Nl}$		0.74		6t·0		0.39				
T 88 b	34-67 34-69	Tb Normal	6 mos.	Increment "		2.0		5.6		13.4				
				Ratio $\frac{Tb}{Nl}$		0.54		0.53		St 0	··	<del></del> ,		
T 88 c	32-86 32-88	Tb Normal	11 mos.	Increment "		3.5		8.0 8.0		17.0	·			
				$Ratio \frac{Tb}{Nl}$		1.52		1.38		1.0			<del></del> -	
Т 89 а	36-25 36-26	Tb	3 wks.	Increment "	1.8	4.6	6.5		9.0	<u>'</u>	11.4	İ		
				Ratio $\frac{Tb}{Nl}$	0.95	1.0	16.0		0.79		09.0			
T 89 b	34-67 34-69	Tb Normal	б тоз.	Increment "	2.2	7.4	11.0		19.2		28.2			
				Ratio $\frac{Tb}{Nl}$	91.1		1.2		1.39		19.0			
Т 89 с	32-86	Tb Normal	11 mos.	Increment "	1.8	5.3	8.7		25.0		58.0			
				Ratio $\frac{Tb}{Nt}$	1.05	8.0	0.87		1.67	<u> </u>	2.23			

tained with the plasma of its normal sibling are given second. ratio Tuberculous plasma is indicated in italics. Excepting in the first experiment (T 37) all the growths were observed for a week or The longer; for it soon appeared that the differences in the influence of the two types of plasma were more evident after a week than during the first few days following transplantation.

The rates of the increment of growth varied widely in the different experiments. This is not surprising in view of the fact that different stocks of transplants were employed at various times. During a certain period the stock was nourished simply with normal plasma, and under these conditions it became evident that the growth energy gradually decreased. With cultures of distinctly poor initial growth energy the depressing influence of tuberculous plasma was so marked that significant growth curves could not be obtained; such experiments were, therefore, eliminated from the final reckoning. When, on the other hand, the stock transplants were nourished with embryonic extract their growth was more vigorous and after being transplanted and subjected to the experimental environments the differences were brought out with significant clarity.

In Experiments T 63 and T 88 a the plasma was obtained on the 18th and 16th days of tuberculosis respectively. It was very turbid and, after cooling and centrifuging, showed a creamy layer. After a few days' growth the fibroblasts nourished in this medium were so filled with yellowish brown granules that they gave the impression of dying cells. They continued to grow, however, and concomitantly the plasma immediately surrounding the transplants became clearer. One seemed justified in concluding, therefore, that the growing cells were phagocytosing lipoidal substances from the tuberculous plasma. With the passage of weeks the plasma from the tuberculous rabbits became progressively clearer, until by the 6th or 7th week it had practically the same macroscopic appearance as normal plasma. During this time, however, the cells growing in the plasma from the diseased animals continued to show a more granular appearance, so that they could easily be distinguished from those growing under a normal environment. Cells grown in tuberculous plasma obtained after the 4th month, on the other hand, had almost the same microscopic

appearance as those grown in normal plasma. The possible significance of these observations is considered in the discussion.

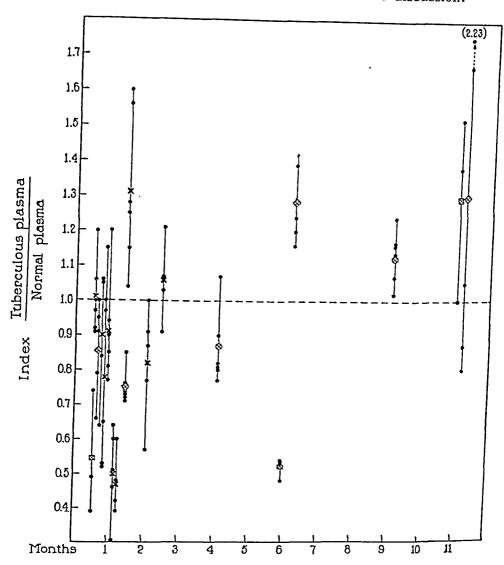


CHART 1. Comparative indices of growth in plasma from rabbits in various stages of tuberculosis.

×'s represent average, and the dots individual observations; the lines indicate the variations in a given experiment.

- $\times$  in circle indicates Experiment T 79, a, b, c, respectively.
- $\times$  in square indicates Experiment T 88, a, b, c, respectively.
- × in diamond indicates Experiment T 89, a, b, c, respectively.

The averages of the ratios Tuberculous plasma compared in graphic form as in Chart 1. These averages, indicated y X, were estimated from all the ratios in each individual experiment; are most easily he range for each experiment is indicated by a vertical line, and each tio is indicated by a dot on the line. Similar symbols are used to dicate experiments where three different plasmas were tested simulcaneously with the same stock of transplants. While this form may not be absolutely correct from a statistical viewpoint, it represents

fairly well the varying influences of tuberculous plasma obtained at different periods in the evolution of the disease in rabbits. This chart demonstrates that most of the average indices obtained with plasma from tuberculous rabbits in the first 3 months of the disease were below 1, while most of those from rabbits having had tuberculosis 6 to 11 months were above 1. Some unexplained exceptions to the rule were observed. In two experiments, Nos. T 68 and T 72, the plasma was 4 days old before it was used for the nourishment of the fibroblasts; in the former the index was always above 1 and was the highest that was seen with plasma in the early period; in the latter the index hovered close to 1. It is possible that during the 4 day period in the ice box the plasma had lost some of its inhibitory Properties. In Experiment T 63, where the index was practically 1, the areas of total growth may have given a false impression of the relative number of cells in the transplants. Microscopic examination showed that most of the cells growing in this very turbid tuberculous plasma were so stuffed with lipoidal granules that the individual cells were nearly twice as large as those growing in the normal control plasma. Had they been the same size probably the areas occupied by the total growths would have been considerably smaller with a corresponding decrease in the index.

In Experiment T SS b the stock of fibroblasts was far from vigorous, so that even a small amount of inhibitory substance in the plasma was apparently reflected in the growth curves. This was shown also in Experiments T 88 a and c that were carried on at the same time. In all instances where the cells were exposed to tuberculous plasma there were distinct microscopic evidences of toxic action of the tuberculous environment. Nevertheless, the differences between the plasmas obtained very early and relatively late in tuberculosis are still brought out in these two sets. In Experiment T 89 the plasma obtained 1 week later from the same rabbits and tested on a new stock of vigorously growing fibroblasts gave results more in keeping with the general rule; here the index with plasma from rabbits suffering from tuberculosis for 6 months was well above 1.

The question naturally arose as to whether the poorer growth in an environment of tuberculous plasma was the result of an increase in inhibitory substances or a diminution in growth-stimulating factors. The fact that, as a rule, the early periods of the tuberculosis occurred

TABLE II

Comparative Rates of Growth of Fibroblasts in Plasma and Scrum of Tuberculous

Rabbits

Experi-	Medium		Day						
ment No.			2	3	4	5	6	7	8
T 66 x	Tb plasma	Increment	1.2	3.6		8.6	16.7		32.0
	Tb serum	"	1.1	4.8		18.0	38.0		66.0
		$Ratio \frac{Plasma}{Scrum}$	1.1	0.75		0.48	0.44		0.49
T 67 x	Tb plasma	Increment	0.58		2.3	5.0		11.0	
	Tb serum	"	1.5	ļ	8.3	16.7		40.0	
		Ratio $\frac{Plasma}{Serum}$	0.39		0.28	0.3		0.28	

during the youth of the animals while the late periods represented maturity suggested that inhibitory substances were responsible for depression of growth; because it is well known that growth-inhibitory substances increase pari passu with age (2). Additional evidence pointing in the same direction was furnished by comparing the action of plasma and serum obtained at the same period from tuberculous animals. The increments in growth and the indices of Plasma are shown in Table II. It is obvious that there were more growth-inhibitory factors in tuberculous plasma than in tuberculous serum. Parallel experiments with normal plasma and serum also showed that the former was much more inhibitory than the latter. Unfortunately,

some of the normal serum controls became contaminated, so that the 159 indices could not be completely estimated. Experiment T 37 (Table I) indicates, however, that serum from rabbits early in tuberculosis is more inhibitory than is that from normal controls. Microscopically the cells grown in tuberculous serum or plasma (Experiments T 66xand T 67 x) became equally granular, much more so than cells grown in normal serum or plasma. Whether this granulation was the result of a toxic action of the plasma on the cytoplasm or of phagocytosed lipoid particles is impossible to state.

## DISCUSSION

Two distinct effects from growing fibroblasts in plasma from tuberculous rabbits were obvious: the first, occurring in the initial stages of the disease, was growth-inhibitory; the second, developing during late tuberculosis, was growth-stimulating. These two effects are roughly parallel with the diphasic nature of tuberculosis observed by Thomas (3) in rabbits inoculated intravenously with the same strain of tubercle bacilli used by us. Because we employed a constant and relatively small dose of tubercle bacilli, and a single breed of rabbits, the course of the infection was fairly constant. With one or two exceptions all of the animals passed into the chronic stage of the disease.2 For this reason it was impossible for us to state absolutelythe type of lesions during the first 3 months, but one may safely conclude that there were extensive diffuse lesions in the lungs, liver, spleen, lymph nodes and bone marrow, and probably in the kidneys. Clinical observations showed that practically all of the males had tuberculosis of the testicles, several had lesions of the joints and eyes; and postmortem examinations of animals dying after 6 to 12 months revealed relatively few lesions in the lungs, spleen, lymph nodes or liver. The genitourinary tract, on the other hand, was almost con-

We are indebted to Dr. R. M. Thomas for several of the cultures used. Much of our work was in progress during some of the period covered by his observations. We feel justified, therefore, in comparing the course of the disease observed in the

Attempts to use a litter of another variety, i.e. English, resulted in early death of all the animals in scalated.

The question naturally arises as to the correlation between the clinical course of the disease and the factors in the plasma that were observed to exert an influence on the growth of fibroblasts in vitro. Serum from cachectic animals is known to contain growth-inhibiting substances; but such elements can hardly be invoked here, because the rabbits continued to gain weight during the first few months following inoculation. True, this gain was not so great as that of their normal litter mates; but, in general, the tuberculous animals appeared to be fairly healthy during this period, and only became cachectic towards the end of their life.

A more probable explanation of the depressing influence of fibroblastic growth observed with plasma in the earlier period of tuberculosis is the presence of an excess of lipoids in these plasmas. was very marked in the 3rd week, and diminished thereafter. Baker and Carrel (4) have shown that the lipoids of serum are the chief growth-depressing components of that fluid; and that the increased growth-inhibiting factors that accompany senescence are associated with an increase of serum lipoids. In our experiments there was no obvious relation between intensity of macroscopic lipoidemia and degree of inhibition. No attempt was made to determine the nature of these lipoids, so one can merely make conjectures concerning their origin. They may have arisen from the diseased tissues, or from the disintegrating tubercle bacilli, or from a combination of both. Other toxic substances may have carried the inhibiting factor, for it is easy to understand how poisons might be released from both pathological tissues and bacterial cells.

The two phases of the influence of plasma on fibroblastic growth may also be compared with the usual course in the blood picture in tuberculous rabbits (Thomas (3)). In the first 2 months there is a leucopenia involving all elements except the monocytes. This is followed by a leucocytosis characterized by a distinct increase in both monocytes and polymorphonuclears. Carrel (5) has designated the leucocytes as the trephocytes of fibroblasts, because growth-stimulating substances can be demonstrated in them. In our experiments growth-inhibitory factors were present in the plasma during the period of leucopenia, and growth-stimulating substances during the stage of distinct leucocytosis. It is, of course, impossible to state

whether the two phenomena have a common basis or are merely concomitant. It is at least of more than passing interest that the stage of tuberculosis in which there is a distinct tendency for the animal to 161 wall off the tuberculous lesions with a thick fibrous capsule should also be characterized by the presence in its plasma of substances which stimulate the growth of fibroblasts to an unusual degree.

Plasma obtained from tuberculous rabbits within 3 or 4 months following inoculation with bovine tubercle bacilli exerted a growthinhibitory influence on transplants of rabbit fibroblasts; while that obtained after the 4th month was growth-stimulating. It is suggested that the growth-inhibitory factor was linked in part with lipoidemia; while the growth-stimulating elements were associated with the period

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# THE FATE OF BCG AND ASSOCIATED CHANGES IN THE

(From The Henry Phipps Institute of The University of Pennsylvania, Philadelphia)

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In view of the widespread use of BCG in the vaccination of infants against tuberculosis, it seemed desirable to determine to what extent this microorganism of low virulence multiplies in the various organs of the body and in the lymphatic tissue, for how long it remains viable within them, and what are the histological changes associated with the fate of the bacilli. Such a study would appear to be prerequisite to an understanding of the capacity of the BCG for protecting animals against a virulent infection. Furthermore, it was hoped that it might throw some light on the pathogenesis of the more complex disease incited by virulent tubercle bacilli, and upon the relation of the tuberculin reaction to the various phenomena observed.

Exhaustive reviews of the vast literature on the BCG have recently appeared (1). Schilling (2) noted clumps of acid-fast bacilli in the lungs of guinea pigs intravenously inoculated with BCG, which he interpreted as evidence of their multiplication. Ninni (3) recovered cultures of BCG from the cervical and bronchial lymph nodes of 3 out of 6 guinea pigs that had ingested the microorganism from 2 to 6 days previously. When emulsions of these nodes were injected into the cervical nodes of normal guinea pigs, acid-fast bacilli could be seen, and, in rare instances, macroscopic colonies were obtained from their lymph nodes from 4 to 8 days after inoculation but not on the 12th day. He concluded that the BCG may multiply for some time in the body.

As to their persistence in the body, Griffith (4) cultured the BCG from the lymph nodes of monkeys 7 months after inoculation, and Birkhaug (5) cultured them from the lymph nodes of guinea pigs

#### Material and Methods

The writer (6) has reported on the fate of a strain of BCG that was fatal to a certain percentage of rabbits. Since the overwhelming majority of studies have demonstrated that the BCG does not cause fatal tuberculosis, another strain, serial No. 376, was brought to this laboratory in 1928 by Dr. S. Mudd directly from the Pasteur Institute in Paris. This strain has been propagated according to Calmette's directions by monthly transplants on veal-glycerol-potato medium, with every ninth and tenth transplant cultured on bile-glycerol-potato medium. Up to this time this BCG has caused no progressive tuberculosis in any of the large numbers of guinea pigs and rabbits into which it has been inoculated.

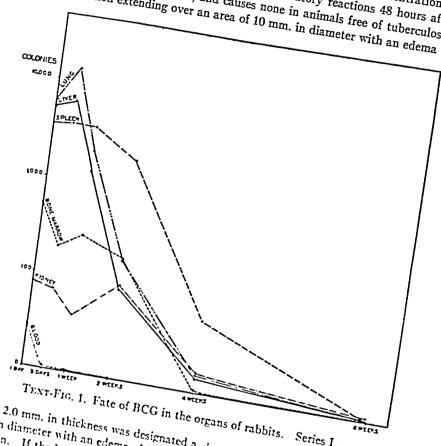
Three series, each consisting of 12 rabbits, were inoculated intravenously with 1.0 mg. of this strain of BCG. 1 and 3 days, and 1, 2, 4 and 8 weeks following inoculation, 2 rabbits of each series were killed; weighed amounts of ground lung, liver, spleen, kidney and bone marrow in various dilutions, and a measured quantity of blood from the heart, were seeded on egg media, directly and after treatment (7) with 6 per cent sulfuric acid. In Series II and III, weighed amounts of ground tracheobronchial and mesenteric lymph nodes, and in Series I, bile from the gall bladder, were also seeded in a similar manner.

Another group of rabbits were inoculated intravenously with 1.0 mg. and placed in a separate room apart from possible tuberculous contagion and kept there as long as 14 months. At the end of this time some of these were killed and cultures were made from the organs and lymph nodes.

In preliminary experiments difficulty was encountered in obtaining consistent colony counts of the BCG on the egg media hitherto used. Ninni (3) and others are of the opinion that there is no suitable medium for the cultivation of the BCG from the body. However it had been noted in previous studies with more virulent bacilli that the colonies that grew on egg media planted with infected bone marrow were more luxuriant than those planted with other infected tissue. Brown (8) also found that the addition of bone marrow infusion to Dorset's medium gave unusually good primary bovine type cultures. Accordingly the number of colonies obtained from seeding a given amount of infected tissue on Dorset's, Petroff's and Löwenstein's media were compared with that obtained on Löwenstein's medium in which bone marrow infusion replaced the distilled water required by the It was found that from 100 to 200 per cent more colonies of BCG, formula (9). and of virulent bovine and human type tubercle bacilli developed on the modified Löwenstein's medium. Therefore this medium, with malachite green substituted for Congo red in 0.034 per cent concentration to suppress contaminants, was used exclusively in this study.

The number of colonies on the surface of each tube from the various dilutions of the different organs was repeatedly determined; the final reading was made after 3 months' incubation. The tissue immediately adjacent to that cultured was studied microscopically after staining with hematoxylin and eosin, and with carbolfuchsin to show tubercle bacilli.

To determine the relationship between the fate of BCG, the histological changes that they produce in the organs, and tuberculin sensitivity, a separate group of 13 rabbits were inoculated intravenously with 1.0 mg. of BCG and at various intervals after infection were tested by the intracutaneous injection of 0.1 cc. of a 1 in 5 dilution of tuberculin derived from the human type. This concentration of O. T. regularly gives rise to well marked inflammatory reactions 48 hours after injection in tuberculous rabbits, and causes none in animals free of tuberculosis. An inflammation extending over an area of 10 mm. in diameter with an edema of



1.0 to 2.0 mm, in thickness was designated a + reaction. An area of redness 20 mm, in diameter with an edema of 2 to 3 mm, in thickness was designated a ++ reaction. If the inflammation extended over an area of 30 mm, in diameter and the edema reached 4 mm, in thickness it was designated a +++ reaction.

Multiplication and Destruction.—In Text-figs. 1 to 3 is depicted the fate of the BCG in each of the three series of animals. Each point

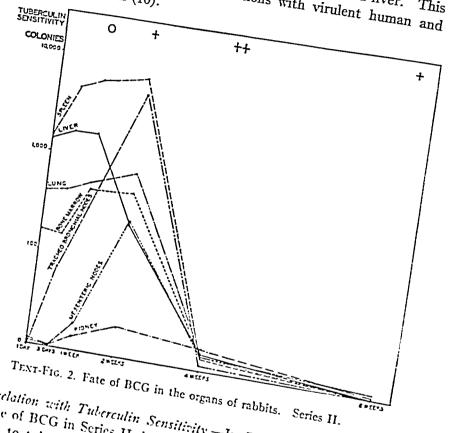
charted represents the average number of colonies obtained from 10 mg. of a given organ or tissue of 2 rabbits inoculated at the same time with the same suspension of BCG. It will be noted that widely differing numbers of colonies were cultured 24 hours after inoculation from the same organs in the three series. Thus in Series I, the primary deposition in the liver and spleen ranged between 3,000 and 5,000, in Series II, between 1,400 and 1,500, and in Series III between 700 and 900. Similar relations obtained in the blood and other organs. Thus 27 colonies were isolated from the blood in Series I, 5 in Series II and 2 in Series III. This difference may be accounted for in part by differences in the amount of moisture contained in the bacillary masses weighed out for each series.

The number of bacilli inoculated as indicated by the number of colonies cultured from the various organs after 24 hours influenced the subsequent fate of the bacilli and the course of the associated changes in the body. With the largest primary deposition, in Series I (Text-fig. 1), multiplication was apparent on the 3rd day in the lung and liver and to a very slight extent in the spleen. By the end of the 1st week there was a marked reduction in the number of bacilli in the lung and liver, while they remained in about the same numbers in the spleen. By the 2nd week the destruction of the bacilli was far advanced in the former organs and had definitely started in the latter. By the 4th week only small numbers of colonies remained in the lung and liver, and the destruction in the spleen was conspicuous, although still lagging. At the end of 2 months the bacilli had disappeared completely from all the organs, with the exception of a rare colony isolated from the kidney.

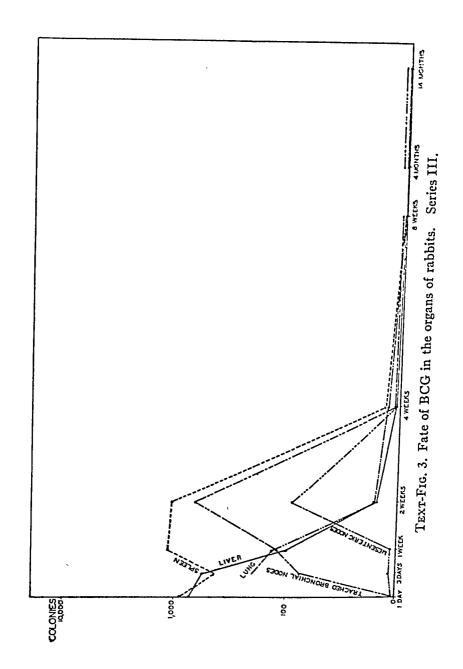
In Series II (Text-fig. 2), where there was a smaller primary deposition of the bacilli than in Series I, growth continued 11 days longer in the lung and spleen and destruction in the liver began a week later. However, again by the 4th week destruction of the bacilli was largely complete in all the organs.

In Series III (Text-fig. 3), where the original deposition of bacilli was smallest, no preliminary growth at all took place in the lung and liver, but destruction began at once, although even in this series conspicuous multiplication occurred in the tracheobronchial and mesenteric lymph nodes, and the bacilli persisted in large numbers in the spleen. They were largely destroyed by the 4th week.

MAX B. LURIE . Thus with a large "dose" multiplication occurs in the first few days and is rapidly followed by destruction. With a smaller dose multiplication continues for a longer time and is followed by a later destruction. With the smallest primary deposition destruction begins at once without preliminary multiplication in the lung and liver. is in accord with previous observations with virulent human and bovine type bacilli (10). COLONIE



Correlation with Tuberculin Sensitivity.—In Text-fig. 2, depicting the fate of BCG in Series II, is indicated the average degree of sensitivity to tuberculin developed by 13 rabbits inoculated with the same strain of bacilli in the same quantity. It will be noted that simultaneously with the appearance of definite skin hypersensitiveness to tuberculin, destruction of the bacilli has started in the liver. By the 4th week, when tuberculin hypersensitiveness has reached its height, the destruction of the bacilli is practically complete in all the



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organs in all the series. Unfortunately the skin sensitivity of the animals whose organs were cultured could not be determined at the time of killing the animal, for 2 days had to elapse between the testing and the reading of the reaction. However it is likely that in Series I, where the primary deposition was much greater, tuberculin sensitivity developed earlier. This follows from the work of Römer and of Lewis and Aronson (11), who found that the tuberculin reaction can be brought about more quickly in animals infected with larger doses of tubercle bacilli than in those infected with smaller doses. The development of skin hypersensitivity to tuberculin is thus synchronous with onset of the destruction of the bacilli.

It is noteworthy, moreover, that even in the relatively susceptible lymph nodes, the bacilli are destroyed at a time when the maximum sensitivity to tuberculin has developed. If tuberculin sensitivity be considered, in part at least, an indication of antigen-antibody reaction, as is widely held, there is thus a consistent correlation between the development of immunity to tuberculosis, as shown by the destruction of bacilli, and the production and accumulation of antibodies. However in the light of these data it can be observed only that the phenomena are parallel, and the one cannot be said to be the cause of the

Relative Immunity in Lymph Nodes and Other Organs.—The behavior of the BCG in the tracheobronchial and mesenteric lymph nodes and in the spleen is deserving of some discussion. It will be noted (Text-fig. 2) that in Series II an almost unhindered multiplication occurred in these lymph nodes at a time when the bacilli were being effectively destroyed in the liver and when the rate of multiplication in the lung was much slowed. In Series III destruction began without any multiplication in the lung and liver, but in the tracheobronchial and mesenteric lymph nodes extensive multiplication continued to the end of the 2nd week, and in the spleen the bacilli persisted without reduction throughout this period. These observations indicate that lymphatic tissue is a peculiarly favorable site for the growth of the BCG, confirming the opinion of Calmette (12).

It might be said that the accumulation of bacilli in the tracheobronchial nodes is ascribable to drainage from the lung rather than to any particular immunity or lack of immunity. But this interpretation cannot be applied to the mesenteric lymph nodes, for the intestinal tract was spared entirely by the infection studied. Moreover the growth and persistence of the bacilli in the spleen cannot be attributed to their segregation and accumulation in this organ from other sources, for the blood stream was always sterile a few days after inoculation. Further evidence was obtained in a study of another series of rabbits also inoculated intravenously with the same quantity of the same strain of BCG. In these animals the same observations were made with the axillary lymph nodes, in which there is practically no possibility of draining bacilli from other organs.

In previous studies (6, 10, 13), the special position of the lung and kidney in regard to immunity developing against a virulent infection has been emphasized. Nevertheless local immunity was not inferred, because it was felt that the multiplication or accumulation of bacilli in these organs when they were being effectively destroyed in the liver, spleen and bone marrow, might be ascribable to some other cause. In the lung and kidney spread through tubules was obviously one important factor. In the lymph nodes, however, this factor also is not operative.

Thus it appears that definite evidence in support of the conception of local immunity is available in the persistence of the BCG in the spleen and their growth in the lymph nodes when they are being effectively checked in other organs. In Series I and II it is apparent that this local immunity is acquired, for at first the bacilli multiply in these organs; later these organs acquire a resistance that destroys the bacilli. For a time the acquired immunity in the lymph nodes and spleen is distinctly less effective than in the other organs. By the 4th week, however, concurrently with the development of marked hypersensitiveness to tuberculin, the bacilli are destroyed almost completely even in the lymph nodes, although isolated living BCG persist in the lymph nodes as late as 14 months after inoculation.

### The Histological Changes

With Multiplication and Destruction.—The tissue responses to the BCG differ in no qualitative way from those incited by bacilli of human or bovine type. The differences, largely of degree, are determined by the limited multiplication and subsequently rapid destruction of the less

virulent, and by the more prolonged multiplication and less efficien destruction of the more virulent microorganisms.

Calmette (12) originally held that the BCG is not tuberculogenic, although retaining the immunizing capacities of the more virulent tubercle bacilli. However Couland (14) showed that large doses intravenously inoculated into rabbits produced typical epithelioid and giant cell tubercles, which regressed without produced typical epithenoid and giant cen tubercies, which regressed without moderation. Schilling (2) found that guinea pigs inoculated intravenously with BCG developed macroscopic tubercles in the lungs. He observed the accumulation of polymorphonuclears about the clumps of bacilli; these cells died, but there was no caseation of tubercles. He noted the encapsulation of the tubercles by fibrous tissue, their gradual shrinkage and final disappearance.

Since the correlation between the fate of more virulent bacilli and the associated histological changes has been described in detail in previous studies (6), only representative animals of the present study will be discussed.

24 hours after inoculation, as in Rabbit 18-06 of Series I, there was a generalized infiltration of the alveolar septa of the lung by polymorphonuclears. Here and there, about clumps of bacilli surrounded by polymorphonuclears. There and were necrotic, there were nodular accumulations of large mononuclears, some of were necrotic, there were nonual accumulations of large mononuclears, some of accumulations of the polymorphonuclears and their wnich had phagocyted the nuclear debris of the polymorphonuclears and their contained bacilli. 7,230 colonies of tubercle bacilli were isolated from 10 mg, of lung of the rabbit described (Fig. 1).

3 days after inoculation, 13,180 colonies were isolated from Rabbit 13-09 of the Same series. There was a moderate number of nodules in the septa and surrounding blood vessels, composed of closely crowded lymphoid mononuclears. In the center of some of these nodules there were large mononuclears with well developed foamy cytoplasms, in which very short acid-fast bacilli might be found. At times these mononuclears formed syncytia, in which the oval vesicular nuclei might these mononuclears formed syncytia, in which the ovar vesicular nuclei inight the periphery of the protoplasmic mass. Mitosis was sometimes seen in these nodules (Fig. 2).

the in these nonlines (rig. 2).

I week after inoculation, as in Rabbit 18-07 of this series, the nodules were I week after inoculation, as in Kabbit 18-0, or this series, the nodines were many of the mononuclears had assumed the typical structure of mature of mature. farger, many of the mononuclears had assumed the typical structure of mature of mature of the colonies (1,900) were isolated. There was as yet no exudation into the alveoli (Fig. 3).

2 weeks after inoculation into the atveolity and of the first series did not Collapse, and were fidded by numerous macro-copic tubercles. Microsporically the tabercles were required by numerous macro-copic tupercles, enterocopically enterocopically into the almost of a standard cells. There was a copspicuous etudation into the alveoli of guil, chitheliola and Langhans plant cells. Encourse exagnition into the alwest of Rull, Gallierona and Language Functions, and following denuclears. At times this exagnition assumed forces. modic proportions. The larger telesceles were infiltrated with polymorphisms.

clears, many of which were necrotic. This acute inflammation was simultaneous with the development of hypersensitivity to tuberculin. Only 30 colonies were recovered from the lung of Rabbit 13-08 (Fig. 4). In the tracheobronchial lymph nodes the first stages of cascation were apparent.

In Series III, in which, as was noted above, destruction of the bacilli was far advanced in 2 weeks, the changes in the lung were essentially those seen in Series I at this interval, and caseous foci were found in the tracheobronchial lymph nodes, as in Rabbit 19-88 (Fig. 10).

In contrast to the changes in Series I at this time was the reaction in Series II, in which, as was noted above, multiplication continued to the 2nd week after inoculation. In the lungs, for example in Rabbit 20-95, there were numerous nodules composed of large mononuclear cells, many of which were in mitosis. Many of the nodules were situated about small blood vessels. On either side of the elastic membrane of some of these vessels there was a large accumulation of mononuclears. These pushed the endothelial lining ahead of them and encroached upon the lumen of the vessel. In some of the nodules were seen young epithelioid cells. Mature epithelioid cells were infrequent. There was no exudation into the alveoli (Figs. 5 and 6). No caseation was noted in the tracheobronchial lymph nodes (Fig. 9). This was the height of multiplication of the bacilli in the lungs of this series; 960 colonies were isolated from the lung described. However when the bacilli in Series II had been destroyed, 4 weeks after inoculation, there was a widespread tuberculosis with exudation into the alveoli.

Essentially the same correlation was found in the other organs. Thus in Rabbit 20-94 of Series II, 1 week after inoculation 11,360 colonies were isolated from the spleen, but except for frequent mitotic figures both in the hypertrophied corpuscles and in the pulp, and collections of large mononuclears in the latter, there was no indication of any tubercle formation (Fig. 7). However by the 4th week after inoculation, when only 1 colony was isolated from 10 mg. of the spleen of Rabbit 44 of the same series, the pulp was largely replaced by large epithelioid and giant cell tubercles (Fig. 8).

Regressive Changes.—All these changes, which at times are extensive, regress and by the end of the 2nd month have largely disappeared. This is brought about by the process of resolution (Fig. 11).

The epithelioid cells become highly vacuolated; they coalesce until only shreds of the cytoplasm remain, although the nucleus retains its viability. 2 months after injection there is almost complete restitutio ad integrum. Only about the blood vessels in the lung and in the portal canals are found collars of large mononuclears (Fig. 12), or there may be found rare collections of epithelioid cells undergoing regressive changes.

There is a complete correlation between the histological changes and the fate of the bacilli as indicated by the number of colonies

cultured from a given tissue. The growth of the bacilli is associated with the accumulation of mononuclears into nodules and their local multiplication by mitosis; the destruction of the bacilli with the formation of epithelioid and giant cell tubercles.

Casealion; Tuberculin Sensilivity.—It is noteworthy that the secondary invasion of the tubercles by polymorphonuclears with their subsequent necrosis, exudation of fluid and cells into the alveoli of the lung, and caseation of the tracheobronchial lymph nodes occur simultaneously, and are synchronous with development of hypersensitiveness to tuberculin. That the first three phenomena are closely bound together is clearly seen by a comparison of the fate of the bacilli and the associated changes in the lung and tracheobronchial lymph nodes 2 weeks after inoculation in Series II and III. In Series II, where multiplication continued for 2 weeks, there is no exudation into the alveoli, there is no invasion of the young tubercles of the lung by polymorphonuclears, and there is no caseation of the tracheobronchial nodes (Fig. 9), although the bacilli are at their height; 960 colonies Were isolated from the lung and 8,360 colonies from the tracheobronchial nodes of one rabbit. In Series III, in which destruction of the bacilli began at once, only 16 colonies were isolated from the lung and 1,020 colonies from the tracheobronchial lymph nodes, but there is an infiltration of the pulmonary tubercles by polymorphonuclears, which are necrotic, exudation of serum and cells into the alveoli, and the tracheobronchial nodes show caseation (Fig. 10).

That the primary toxicity of tubercle bacilli is not responsible for these acute inflammatory changes is clearly indicated by the fact that in the spleen and in the lungs the bacilli may be present in great numbers 3 days and 1 week after inoculation without any of these changes; later, simultaneously with the development of hypersensitiveness to tuberculin, the presence of far fewer bacilli is associated with necrosis of invading polymorphonuclears, exudation into the alveoli and cascation in the lymph nodes.

It was noted above that the lymph nodes are a particularly favorable site for the growth of the BCG; that the immunity developed in them is at first less effective than that of other organs. Therefore it is understandable that cascation develops in them, whereas only necrosis of polymorphonuclears is observed in the lung. When hypersensitiveness is sufficiently developed too few bacilli remain in the lung and liver to exercise in a sufficient degree their newly enhanced toxic effect on the tissues, but in the lymph nodes, where enough bacilli remain even after development of hypersensitiveness, there is not only death of the invading polymorphonuclears but also necrosis of the epithelioid cells and caseation.

Caseation and tuberculin sensitivity thus bear the same relation to the course of the disease. As the disease progresses tuberculin has a more toxic effect and at the same time the presence of relatively small quantities of bacilli in the tissues is associated with caseation. In an earlier stage of the disease much larger numbers of bacilli do not injure the tissues, and the same or a larger quantity of tuberculin does not cause inflammation.

It is evident, therefore, that the BCG produces the same histological changes in the tissues that more virulent tubercle bacilli do. The differences are due to the restricted multiplication and more rapid and complete destruction of the BCG, associated with the limited development of mononuclear infiltrations, their rapid transformation into epithelioid tubercles and their subsequent complete resolution. Only one phenomenon observed in virulent infection is lacking with the BCG; that is, caseation does not go on to softening.

Caseation, which, it has often been asserted, does not develop with the BCG, was regularly found in the lymph nodes, where the bacilli persist the longest. Softening, as is well known, develops only during a protracted course of a virulent infection. Since the BCG all but completely disappeared from the body in 2 months and the pathological changes, including the caseation that they had induced, completely resolved, it is obvious that softening could not take place.

## The Persisting Bacilli; Disposal of the BCG

It has been seen that in the mesenteric and tracheobronchial lymph nodes and occasionally in the spleen and kidney isolated bacilli were cultured even 14 months after inoculation. At this time the organs were entirely normal in their histological structure. It was found, moreover, that after their long sojourn in the body, the BCG recovered from these organs had acquired no increase of virulence for rabbits.

Destruction of the BCG appears to occur in situ. Certain ob-

servers have concluded that bacilli of reinfection are eliminated from the body with the bile (15). However in the present study the BCG were never cultured from the bile except during the first days after 175 inoculation. At that time they could be accounted for by contamination with blood, in which they were then present in considerable numbers.

For some time after intravenous inoculation into rabbits the BCG multiplies in various organs of the body. As with more virulent tubercle bacilli, the greater the primary deposition of bacilli the more rapid is their initial growth and the earlier is the beginning of their destruction. Destruction sets in synchronously with the development of definite hypersensitivity to tuberculin. With the smallest deposition destruction begins at once in the lung and liver without preliminary multiplication. However multiplication always takes place in the lymph nodes. It continues here and in the spleen at a time when other organs have acquired sufficient immunity to reduce the number of bacilli considerably. It is seen that the relative susceptibility of the lymphatic tissue is not due to such factors as drainage. Thus the local immunity acquired by the lymph nodes against the BCG is for a time less effective than that developed in the other organs. However by the end of the 4th week, simultaneously with the development of marked hypersensitivity to tuberculin, even these structures acquire sufficient immunity to bring about the all but complete annihilation of the bacilli. Isolated microorganisms persist in the lymph nodes even 14 months after inoculation. These bacilli do not acquire any added virulence because of their long residence in the

As observed with more virulent strains in previous studies, the growth of the bacilli is associated with a local accumulation of mononuclears into nodules and their multiplication by mitosis, the destruction of the bacilli with the formation of epithelioid and giant cell tubercles. These changes may be extensive and at times involve a large part of the lung and lymph nodes. The secondary invasion of the tubercles by polymorphonuclears, their death, exudation of scrum and cells into the alveoli of the lung, and cascation in

the lymph nodes occur simultaneously and are synchronous with the development of hypersensitiveness to tuberculin. At this time the presence in the tissues of numbers of bacilli that previously were innocuous to the cells is associated with caseation. It is noteworthy that both caseation and the tuberculin reaction are not elicited in an early stage of the disease, but in a later stage they are induced in response to smaller quantities of bacilli or tuberculin.

All the tuberculous changes, including the caseous foci in the lymph nodes, usually resolve completely by the end of the 2nd month after inoculation without leaving any fibrous scars, although perivascular accumulations of mononuclears may still remain. The few bacilli that persist in the lymph nodes after this period cause no tuberculous changes.

#### CONCLUSIONS

- 1. Under the conditions of experiments in which 1.0 mg. of BCG was introduced intravenously into rabbits, the BCG multiply in the body, but they are soon destroyed, completely in most organs, all but completely in the lymph nodes.
- 2. The remaining BCG persist in the lymph nodes for a long time, causing no tuberculous changes, and acquiring no added virulence for rabbits.
- 3. The BCG produce typically tuberculous changes, sometimes extensive, which resolve completely. They caused caseation, but no softening.
- 4. Acquired local immunity in tracheobronchial, mesenteric and axillary lymph nodes and in the spleen is shown to be less effective for a time than that in other organs.
- 5. The destruction of bacilli begins with the appearance of skin sensitivity to tuberculin, and is at its height with maximum sensitivity.
- 6. The secondary acute inflammatory reactions in and about tuberculous foci, caseation and hypersensitivity to tuberculin develop synchronously.
- 7. Caseation and tuberculin sensitivity do not occur early in the course of the disease in response to considerable amounts of bacilli and of tuberculin, but later they are incited by smaller amounts.

Acknowledgment is made to Miss Eleanor S. Cooper of The Henry Phipps Institute for editorial assistance.

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All sections were prepared from tissues stained either with hematoxylin-eosin or by the Ziehl-Neelsen method, and counterstained with hematoxylin. The

- Fig. 1. Lung of Rabbit 18-06 of Series I, 24 hours after inoculation; 7,230 colonies were isolated from 10 mg. of tissue. The alveolar septa are infiltrated by polymorphonuclears. About a clump of bacilli not shown in figure are necrotic polymorphonuclears surrounded by a nodular accumulation of mononuclears.
- Fig. 2. Lung of Rabbit 13-09 of Series I, 3 days after inoculation; 13,189 colonies were isolated. A nodule composed of dosely crowded lymphoid mononuclears, in the center a syncytium with large oval vesicular nuclei.
- Fig. 3. Lung of Rabbit 18.07 of Series I, 1 week after inoculation; 1,900 colonies were irolated. The nodule is larger than in Fig. 2. Many of the mononuclears have assumed the typical structure of mature epithelioid cells. X2%,

Fig. 4. Lung of Rabbit 13-08 of Series I, 2 weeks after inoculation; 30 colonies were isolated. Widespread tubercle formation with exudation of fluid and cells into the alveoli. ×100.

#### PLATE 7

- Fig. 5. Lung of Rabbit 20-95 of Series II, 2 weeks after inoculation. The bacilli have attained their largest numbers; 960 colonies were isolated. Numerous mononuclear nodules are seen. There is no exudation into the alveoli. ×100.
- Fig. 6. A higher magnification of another portion of the section shown in Fig. 5. A perivascular nodule with a large accumulation of mononuclears on either side of the elastic membrane. There are no mature epithelioid cells. To the right and above the elastic membrane is a mitotic figure. ×200.
- Fig. 7. Spleen of Rabbit 20-94 of Series II, 1 week after inoculation; 11,360 colonies were isolated. There is no tubercle formation. A mitotic figure can be seen in the pulp in the center of the photograph. ×200.
- Fig. 8. Spleen of Rabbit 44 of Series II, 4 weeks after inoculation; 1 colony was isolated. Extensive tubercle formation in the pulp. ×200.

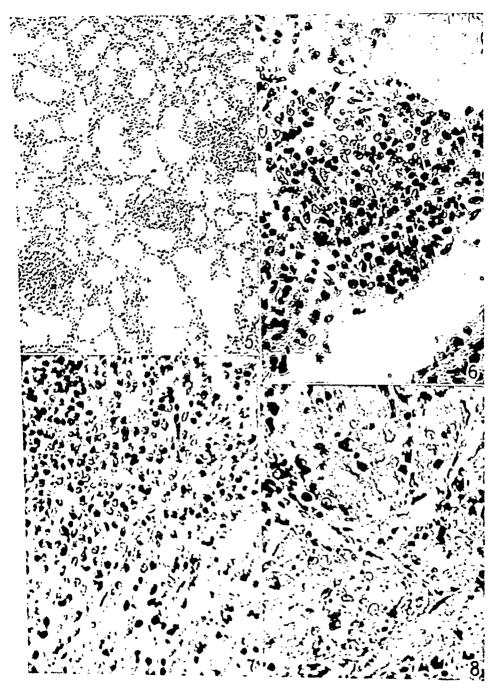
#### PLATE 8

- Fig. 9. Tracheobronchial lymph node of Rabbit 20-95 of Series II, 2 weeks after inoculation; 8,360 colonies were isolated. There are islands of young epithelioid cells. A mitotic figure can be seen in the upper third of the photograph. There is no caseation. ×200.
- Fig. 10. Tracheobronchial lymph node of Rabbit 19-88 of Series III, 2 weeks after inoculation; 1,020 colonies were isolated. Cascation and edema of surrounding epithelioid cells. ×200.
- Fig. 11. Liver of Rabbit 30 of Series III, 4 weeks after inoculation. 3 colonies were isolated. A resolving tubercle. ×200.
- Fig. 12. Lung of Rabbit 34 of Series III, 2 months after inoculation. No tubercle bacilli were cultured. A perivascular collar of mononuclears. × 200.



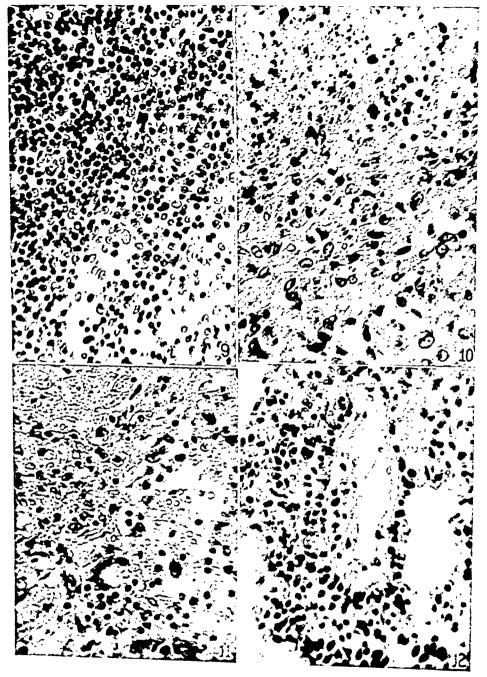
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## RESULTS OF IRRADIATING STAPHYLOCOCCUS AUREUS BACTERIOPHAGE WITH MONOCHROMATIC ULTRAVIOLET LIGHT

By FREDERICK L. GATES,\* M.D.

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(Received for publication, May 24, 1934)

Ever since d'Herelle's announcement of the discovery of lytic principles for bacteria now collectively called bacteriophage, interest and debate have centered on the nature of the active agent. main points of view and bases of contention relate bacteriophages respectively to living organisms (ultramicroscopic viruses parasitic on living bacteria), and to agents of unknown but simpler structure, originating in the bacteria themselves and affecting living bacteria by direct chemical, possibly enzymatic or catalytic, action.

It has occurred to a number of investigators to seek evidence as to the nature of bacteriophage by a study of its reaction to ultraviolet light, in the hope that a direct comparison with the bactericidal action of ultraviolet energy would show significant similarities or differences. In each instance the results have been disappointing. Though it has been shown repeatedly that bacteriophages are destroyed by exposure in broth, in salt solution, or on agar plates to the total or filtered radiations of the sun or of artificial light sources, no critical estimates of the incident energies involved or of the effective wave-lengths have been undertaken. Without describing in detail the methods used or the results obtained, for example by Zoeller (1), Gerretsen, Gryns, Sacks, and Sohngen (2), Gildemeister (3), Biemond (4), or Appelmanns (5), one may conclude with the authors themselves that their studies were not sufficiently critical to give an unequivocal result. Usually the only estimate of the energy involved in inactivation of phage has been a comparison with the time required to sterilize a bacterial suspension,

<sup>\*</sup> This paper is one of several in which are presented results of work completed by Dr. Frederick L. Gates before his death on June 17, 1933. The manuscripts have been prepared by Professor W. J. Crozier and Dr. R. H. Oster.

and such ratios range from 1:1 (Gildemeister (3)) and 1.5:1 (Zoeller (1)) to 8+:1 (Gerretsen (2)), after exposure to the entire spectrum.

But even if the total incident energies had been measured with thermopile and galvanometer, and if quantitative methods of estimating the percentage destruction of both bacteriophage and bacteria had been available (6), such figures would be useless for comparison unless the energies involved at single wave-lengths were determined. Different wave-lengths in the ultraviolet have qualitatively and quantitatively very different specific actions on biological materials, depending on specific absorption and the resulting photochemical reactions. It can hardly be overemphasized that for analytical studies of these reactions monochromatic energy must be used.

Recently we have been engaged in an extended study of the bactericidal action of ultraviolet light, using monochromatic radiations and measured energies (6). It was thought that these quantitative observations on S. aureus and B. coli might serve as a basis of comparison in a more critical examination of the reactions of its specific bacteriophage. A quartz monochromator (6), with thermopile and galvanometer for energy measurements in absolute units, served to bring ultraviolet radiations of known wave-length and incident energy to the plane of exposure. But many difficulties were experienced in devising a method for a quantitative estimate of inactivation of phage. The impossibility of equal, coincident exposure of all the units in a fluid medium required the distribution of the bacteriophage on a plane surface similar to that of the control bacteria on agar plates. Hence the destruction of phage was to be estimated by counting plaques in exposed and in unexposed, control areas rather than by dilution limits.

#### EXPERIMENTAL

In the earlier experiments phage and bacteria were mixed in suspension, washed on to the agar surface, and exposed coincidentally. With suitable concentrations of phage and bacteria discrete colonies developed in the control areas and all showed partial or complete lysis after 18 to 42 hours' incubation. In the exposed areas partial or complete destruction of the bacteria occurred. When many bacteria survived and subsequently formed colonies, some showed lysis and some did not, indicating partial destruction of the phage also. It was typical to find that when but a few colonies of *S. aureus* developed, they were at first apparently free from phage, and in the early hours of incubation it appeared that the phage was the

more susceptible. But when incubation was prolonged, so that the surviving colonies spread more widely over the exposed area, lysis almost always began colonies spreau more wheely over the exposed area, tysis almost always oegan locally at the edges. This was interpreted to mean that in other instances phage had survived when the particular bacterium on which it had been adsorbed had succumbed either to lysis or to ultraviolet exposure. Consequently, no conclusion could be drawn as to the relative susceptibility of bacteria and bacteriophage from such experiments.

It was then proposed to distribute the phage alone on the agar surface, to expose areas to the ultraviolet light, and to wash or sprzy a suspension of S. aureus on the agar to serve as an index of survival of phage by plaque formation. Re-Peated attempts to obtain an even distribution of bacteriophage by washing the gar surface with dilute phage suspensions proved the method unsatisfactory. Finally, it was found that unless the phage was actually in contact with a surface layer of bacteria, plaques were not formed. Thus, phage could be mixed with the nutrient agar before plates were not tormed. Inus, phage could be mixed with the quently sprayed on, only those units of phage which were on the surface gave rise to plaques. That no appreciable extension of deeper phage units to the surface occurred was shown by the fact that a drop of plain agar at 39°C. pressed out in a very thin layer on the phage-covered surface showed no plaques by diffusion through its substance, and even a simple rinsing of the surface of the phage-agar with broth or a bacterial suspension reduced the subsequent plaque formation almost to zero. After irradiation of the phage-agar plates, S. aureus had to be sprayed on to the level surface in a fine mist, lest the surface units of phage be displaced. Then a covering layer of viscid, quick setting agar at 39°C. could be poured slowly over the surface as in the bactericidal experiments (6), without dis-

poured slowly over the surface as in the bactericidal experiments (o), without distributing either bacteria or phage. So the method finally adopted was as follows: The strain of S. ourcus used previously in the bactericidal studies, and its through his kindness, were employed in the present investigation. Bacteriophage through his kindness, were employed in the present investigation. Bacteriophage suspensions, intered inrough Chamberland inters, with a tire of 10-10, were so missed with nutrient broth as to produce discrete, easily counted plaques when mixed with nutrient 2 per cent agar and poured in small Petri plates. As already. Stated, these plaques developed only in the agar interface after the surface had been stated, these plaques developed only in the agar interaceatter the surface had been sprayed with a heavy suspension of an 18 hour culture of S. aureus and covered with a second layer of sterile agar at 39°C.

After the phage-agar had set, and before the S. aureus was sprayed on to serve After the phage-agar had set, and before the standards was sprayed on to serve phage, but spread with S. aureus as in the bactericidal study (6) for comparison Pringe, but spread with a carear as in the decience at study (0) for comparison, as the first successful with single wave-lengths of measured ultraviolet energy in timed were irradiated with single wave-lengths of measured ultraviolet energy in timed each national concerns of four similar sense of cir assured ultraviolet energy in timed cach expensive and state two experiments were performed at each wave-leavily and expensive two per plate. The expensel place place were then sprayed with a heavy surpension of S. owens. The expension place places were then sprayed with a nearly surjection of a covered of the expension of a covered of the place place and the expension critical of the expension of a covered of the expension of the expension of a covered of the covered of the expension of a covered of the expension of a cov Plates of S. current non-the and temp the Power Dates and the exposed control to business a temporal fronth and temp the Power Dates and the exposed control to business a temporal force.

night at 37.5°C. Then the plates were set up in a mechanical stage under a microscope, and plaques or colonies were counted in definite areas between parallel lines and between stops on the mechanical stage. Three control counts in unexposed areas were alternated with the counts of the two exposed areas on each plate and destruction of phage or of bacteria was estimated by comparison. Some unevenness of plaque distribution introduced appreciable errors in the control counts, which tended to cancel out as the number of observations at each wave-

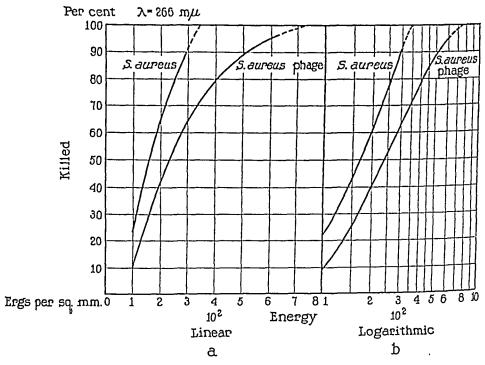


Fig. 1. A comparison of the bactericidal action and destruction of bacteriophage (S. aurcus) at wave-length 266 m $\mu$ . The curves are each the averages of smoothed curves from two experiments and have been extrapolated from 92 per cent and from 97 per cent respectively to 100 per cent destruction.

length increased. Relatively smooth curves could therefore be drawn through the points of observation.

Thus at each wave-length a series of readings was obtained, showing progressive inactivation of phage with increasing exposure. At the same time a direct comparison could be made with the effects of similar exposures on *S. aurcus* in bactericidal tests under similar experimental conditions.

#### DISCUSSION

Fig. 1a shows the bactericidal action and the destruction of phage at  $\lambda$  266 m $\mu$  in a summary of typical results. The curves are each

the averages of smoothed curves from two experiments (cf. 6) and have been extrapolated from 92 per cent, and from 97 per cent, to 100 183 per cent destruction, respectively. Although the reaction curves do. not run strictly parallel, their general shape is the same: the bacteriophage units required somewhat more energy for their destruction than did the bacteria themselves. The energy relationship is best shown by plotting the energy logarithmically (Fig. 1b).

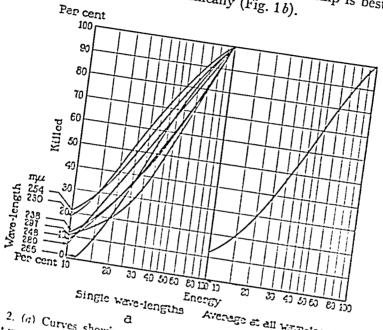


Fig. 2. (a) Curves showing the inactivation of S. aureus bacteriophage at different wave-lengths. For comparison all of the curves have been reduced to the same energy scale by figuring the ratios of the energies involved in partial destruction to those for total destruction and plotting the resulting curves together. In (b) all curves are averaged to give the average shape of the reaction curve.

Except for differences in the incident ultraviolet energy involved at different wave-lengths, similar curves were obtained with the results at  $\lambda$  207, 280, 253, 248, 238, and 230 m $\mu$ . In each experiment essentially similar curves, at somewhat different energy levels, were found for bactericidal action and destruction of phage. Since the energies required for complete phage destruction range from 710 (\lambda 266) to 7.250 (A 207) Circ pers 1, mm, of exposed area, it is not convenient to

assemble them on a single chart, but they may be more closely compared by computing the ratios of the energies involved in partial destruction to those for total (100 per cent) destruction and plotting the resulting curves together, as in Fig. 2a. Some of the curves are convex above and some below the average line, indicating that these variations are indifferent and due to experimental errors. When the curves are averaged, as in Fig. 2b, the errors appear to cancel out, and through most of its course the curve shows a true exponential

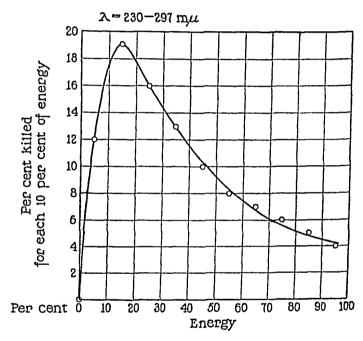


Fig. 3. Curve showing the variation in resistance of *S. aurcus* to different incident energies of ultraviolet radiation. The points are plotted from the data of Fig. 2b refigured as a curve of probability.

relationship of energy to effect. Were the line straight, it would indicate that no phage was destroyed until 10 per cent of the energy required for total destruction had been involved in the reaction. The deviation, therefore, points to the presence of less resistant units of phage, which are destroyed early. In the bactericidal study of S. aureus (6) a similar deviation at the beginning of the curve of destruction was likewise attributed to the early loss of less resistant organisms, but could result from the presence of clumps of the bacteria. Experi-

ments of d'Herelle (7) have indicated that differences occur in the resistance of phage units to harmful agents.

On the other hand, if the particulate nature of bacteriophage be emphasized as a basis of interpretation, the entire course of the curve can be postulated as one of probability (Fig. 3), due to continuous variations in the resistance of the particles over the entire range of

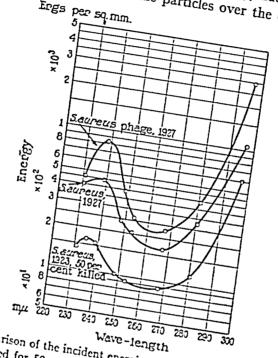


Fig. 4. A comparison of the incident energies per square millimeter of exposed agar surface required for 50 per cent inactivation of phage and for bactericidal action on 50 per cent of S. surcus. The points are plotted from the data of Fig. 2

action of the monochromatic light. But because of errors of distribution in the plates, the early portion of the curve is the least accurate, and we prefer not to lay stress either upon its deviation from a straight line or, on the other hand, upon its general similarity to the curves for monomolecular chemical reactions. It should be emphasized that the curves in Figs. 1 to 3 indicate only the general course of the reaction and give no evidence of the mechanism involved. At the present

state of our knowledge similarities either to probability curves or to monomolecular reaction curves have no determinative significance.

What is more important at present is a demonstration of the energies at different wave-lengths that are required to effect a similar destruction of phage, and a comparison of these energy values with those for a corresponding destruction of S. aureus. For this purpose the incident energies per square millimeter of exposed agar surface required for 50 per cent inactivation of phage and for bactericidal action on 50 per cent of S. aureus have been read from the curves and are plotted in Fig. 4. The similarity of the curves is striking and can only be due to an essential similarity of the underlying reactions. Stress is laid on the practical identity of the curves in shape, rather than on the relative energies, in ergs, involved in the two reactions. The energy values obtained in any one series of observations depend on certain experimental conditions which vary in different series of experiments, though they may be held practically constant over a given period of observation. For example, we have included in Fig. 4 two curves for 50 per cent destruction of S. aurcus. One was obtained in 1923, the other is from the control data of the present series. We do not know why the incident energies required in 1923 are different from those involved in 1927. But what is essential is the similarity in shape of the bactericidal curves, and the relation to one another of the energies required at different wave-lengths. So with the curve for bacteriophage inactivation, the relative, and not the absolute energy values at different wave-lengths, and the similarity in the shape of the curve to that for bacterial destruction are its significant features.

Something of the significance of the bactericidal curves is already known (8). They are almost the reciprocals of those for the specific absorption of ultraviolet light by protoplasm, by proteins, by certain amino acids and nucleoproteins (8), and by certain enzymes (9). It is axiomatic that specific light absorption is fundamental to the reaction of such biological materials to ultraviolet light.

Unfortunately we have no method of correcting the incident energies for the destruction of phage by the absorption coefficients for bacteriophage at different wave-lengths, whatever they may be, for specific light absorption is masked by the presence in the phage suspensions of light-absorbing materials from the nutrient broth and the

lysed bacteria. But even without having the absorption coefficient available for purposes of correction, the similarity of the incident energy curves for inactivation of phage and bactericidal action permits 187 a clear conclusion to be drawn: the essential reactions that result in the death of bacteria and in inactivation of phage follow similar paths.

When, however, a deduction as to the nature of bacteriophage is attempted from this fact, it is evident that more than one interpretation is possible. The simplest and most obvious would be that bacteriophage is essentially of the same nature as bacteria; i.e., that it is a microorganism (7, 10), containing the same light-sensitive elements as do bacteria, and reacting to exposure by the same photochemical processes. A related possibility would postulate that bacteriophage may not be living protoplasm (11), but some highly organized product of bacteriolysis (12), chemically similar to certain constituents of protoplasm essential to life, with similar absorption coefficients and similar ultraviolet susceptibility. Since proteins and some amino acids and enzymes have absorption curves almost the reciprocals of bactericidal incident energies, the present evidence does not permit a choice between these two hypotheses.

Such interpretations and conclusions have not been acceptable to all of those with whom we have discussed the matter. An alternate interpretation is also based on the inevitable presence in the exposed phage suspensions of the products of bacteriolysis. It might be assumed that it is indeed these products whose specific ultraviolet absorption and photochemical reaction are represented by the curve of inactivation of phage. But the phage itself may be of entirely unknown nature, and of unknown ultraviolet absorption and susceptibility. It may be so intimately united with these products of bacteriolysis that the relationship is essential to the activity of phage and a change in this relationship by photochemical change in the bacterial element even though the phage element be unaffected may result in total and irremediable phage inactivation.

The incident energies required to kill Staffigheoccus aureus or to inactivate its homologous bacteriophage have been measured at the various wave-lengths of the quartz mercury vapor are between 258

and 302 m $\mu$  and found to run strictly parallel, the readings for the S. aureus phage being obtained at a uniformly higher energy level. This difference in levels is of less significance than the striking similarity in the shapes of the energy curves, which indicate that in both instances the same organic structures are absorbing the radiations. The results are open to three interpretations. The most obvious is that the bacteriophage is a submicroscopic organism. Again, it is possible that the bacteriophage is a product of its own lytic action on the homologous bacterium and contains the essential structural units which in S. aureus also are destroyed by ultraviolet light and thus cause the death of the organism. A third, more remote explanation is that the phage, of wholly unknown nature, is absorbed on S. aureus material in so intimate a bond that the alteration of this material by irradiation renders the phage incapable of further lytic activity.

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## FATE OF BILIRUBIN IN THE SMALL INTESTINE\*

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(Received for publication, May 21, 1934)

The question of the existence of an enterohepatic circulation of bile pigment has been a subject debated for the last 18 years. The main proponents of the existence of an enterohepatic circulation of bile pigment are Broun, McMaster and Rous (1) and McMaster and Elman (2), while on the other hand Whipple (3) has consistently expressed his disbelief in such a mechanism. In 1923 Broun, McMaster and Rous (4) published data from which they concluded that such a circulation existed. Two years later McMaster and Elman (2) published additional data to further substantiate this theory. The latter authors reported that bilirubin or urobilin either in pure solution or in bile, when introduced into the duodenum of bile fistula dogs, caused an increase of the total bilirubin excretion by the liver.

Whipple (3) doubted the existence of an enterohepatic circulation of bile pigment on the basis of insufficient evidence showing that either bilirubin or urobilin was actually absorbed from the intestine.

The opportunity presented itself of investigating the fate of bilirubin in segments of intestine in animals with modified Thiry (5) loops. In order to compare results from a segment of a physiological unit with the entire unit, the fate of bilirubin in the intestine of the unanesthetized dog has also been studied. Since the chief objection to the acceptance of the concept of an enterohepatic circulation lies in the lack of quantitative data showing that bilirubin is absorbed from the intestine, it seemed of importance to carry out studies to determine this point.

Aided by a grant from the Faculty Research Committee of the University of Pennsylvania.

#### Method

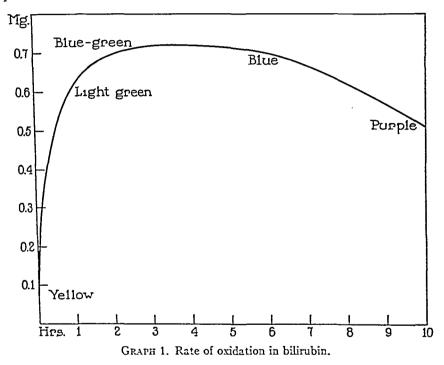
The animals used in these experiments were healthy dogs in whom a modified Thiry (5) loop had been prepared after the method of Johnston (6) some months previous to the experiment. This method permits of quantitative studies in unanesthetized animals. The nerve, blood and lymphatic supplies of these loops were intact and frequent studies of the rate of absorption of glucose indicated that the loops were functioning in a constant manner. It was only necessary to introduce a known amount of pigment into the loop and after a period of time had elapsed to remove all the fluid remaining and determine the pigment which was recovered. The loops were never sterile, but repeated irrigation results in a loop which is free from the usual organisms normally found in the intestine. Before any material was introduced into the isolated loop the latter was thoroughly cleansed. The material introduced into the loops for study was prevented from escaping by a special balloon catheter devised by Johnston (6). No anesthesia was necessary at any time.

The animal whose entire small intestine was to be utilized for absorption was operated upon under ether a week before the absorption experiment was to take place. Two catheters were introduced into the common duct, one of which was directed toward the liver and the other was directed toward the duodenum. By this procedure it was possible to collect all the bile and thus have the small intestine free from any bile pigment. In order to further insure that no pigment remained the animal was given a dose of magnesium sulfate 2 days before the experiment.

Food was withheld for 24 hours before the experiment began although the dog was allowed to have all the fluid desired. Known quantities of liver bile were introduced into the duodenum of the animals through the catheter which was directed toward the duodenum. The bile was allowed to remain in the intestine for a period of 1 hour at which time the animal was sacrificed. The intestinal tract was then rapidly removed from the animal and flushed first with water and then alcohol. These washings were immediately analyzed for bilirubin. The contents of the stomach were tested for bile pigment in order to ascertain whether any regurgitation of bile took place. If regurgitation had occurred the experiment was discarded. By this method it was possible to obtain almost total recovery (mean 97.5 per cent) of pigment immediately after the introduction of the bile.

The bile used was obtained from bile fistula dogs intubated by the double intubation method of Elman and McMaster (7). By this method it was possible to keep the animal in excellent condition since bile was obtained only at intervals and for a long period was rediverted into the duodenum. Daily bacteriological studies were made on the bile to ascertain whether it was sterile before being used. Bile placed in unsterile containers was labeled as contaminated bile. The infecting organisms were the streptococcus and staphylococcus. When grossly infected bile was desired normal saline solution containing colon bacilli was injected into the catheter directed toward the liver.

The Estimation of Bile Pigment.—The bilirubin was quantitatively determined by a modification of the Hooper and Whipple (8) method. A slightly stronger solution of acid-alcohol (0.40 cc. concentrated nitric acid and 6.00 cc. concentrated hydrochloride in 100 cc. 95 per cent ethyl alcohol) was used for the oxidation of the bilirubin. The increase in the acid concentration increased the speed of oxidation and this is further enhanced by keeping the solution at 37.5°C. instead of at room temperature which was the method outlined by Hooper and Whipple (8). By this procedure a greenish blue color develops which may then be compared with a standard color solution in a Duboscq colorimeter. The standard



was prepared by the addition of 0.75 cc. of a 1 per cent potassium dichromatic solution to 100 cc. of a 10 per cent copper sulfate solution. This standard was found to represent the color developed by 0.181 mg. bilirubin per cc. Various dilutions of the standard were utilized.

In the determination of bilirubin by this method, readings were made hourly after mixing the specimen of bile and acid-alcohol together until a maximum color concentration was reached. The maximum color usually developed within 4 hours. Once this color was reached it remained constant for several hours and then the deep greenish blue color changed to blue, purple and finally to black. Graph 1 indicates the rate of color change as compared with the standard solution.

In order to ascertain the experimental error of this method a weighed amount of bilirubin (Eastman Kodak Company) was dissolved in a known amount of 0.05 per cent sodium hydroxide solution. The concentration of bilirubin per cubic centimeter of solution was then calculated. A definite volume of the solution was then oxidized until the greenish blue color was reached and colorimetric determination made. The error of the method did not exceed 10 per cent.

#### RESULTS

Effect of Intestinal Juice on Bile Pigment in Vitro.—Before beginning the experiments on the absorption of bile pigment from the

TABLE I

Absorption of Bilirubin from Sterile Bile in Entire Intestinal Tract

1 Hour's Duration

Experiment No.	Amount in		Amount out	Loss		
	cc.	mg.	mg.	nıg,	per cent	
1	40.00	42.80	15.20	27.60	64.5	
2	40.00	42.80	15.74	27.06	63.2	
3	40.00	36.40	12.87	14.34	39.3	
4	40.00	25.87	25.87	10.53	28.9	
5	40.00	32.00	18.06	13.94	43.6	
6	40.00	32.00	16.09	15.91	49.7	
7	37.00	13.22	8.60	4.61	34.9	
8	50.00	88.00	56.32	31.68	36.0	
9	50.00	88.00	66.72	21.28	24.2	
10	50.00	88.00	44.79	43.21	49.1	
11	40.00	51.60	34.12	17.48	33.9	
12	50.00	64.50	27.59	36.91	57.2	
Average			·		43.7	

intestinal loop it seemed important to know whether the intestinal juice secreted into the loop had any effect per se upon the bile pigment which would prevent good recoveries.

A known quantity of bile was mixed with some intestinal juice obtained from the loop. This solution was placed in a large test tube and kept at a temperature of 37.5°C. for a period of 2 hours. In one instance 1 cc. of the bile used for these studies contained 0.53 mg. of bilirubin before being mixed with the intestinal juice. After the 2 hour period 0.53 mg. of bilirubin was recovered. In none of these

TABLE II a

Absorption of Bilirubin from Sterile Bile in Intestinal Loops
2 Hours Duration

Experiment No.	Amount in		Amo	unt out	Loss		
	cc.	mg.	cc.	mg.		1 .	
1	25.00	19.00	53.00	20.93	mg.	per cent	
2	25.00	19.00	39.00	20.93	+1.93	+10.1	
3	25.00	19.00	130.00	18.98	+2.25	+11.7	
4	25.00	19.00	68.00	20.06	0.02	0.1	
5	25.00	19.00	76.00	15.65	+1.06	5.5	
6	25.00	19.00	225.00	18.90	3.35	17.6	
7	20.00	16.00	58.00	17.98	0.10	0.5	
8	20.00	16.00	40.00	17.98	+1.98	+12.4	
9	20.00	16.00	180.00	15.48	+1.20	+7.5	
10	25.00	27.00	65.00	1	0.52	3.3	
11	25.00	27.00	58.00	24.05 25.24	2.95	10.9	
12	25.00	27.00	190.00	28.12	1.76	6.5	
13	20.00	27.00	70.00	26.60	+1.12	+4.1	
14	20.00	27.00	185.00	28.49	1.20	4.3	
15	15.00	12.00	150.00	13.50	+0.69	+2.5	
16	20.00	18.60	26.00	18.59	+1.50	+12.5	
17	20.00	18.60	50.00	18.75	0.01	0.0	
18	18.00	31.68	105.00	29.82	+0.15	+0.8	
19	25.00	44.00	74.00	41.44	1.86	5.8	
20	25.00	44.00	36.00	39.60	2.56	5.8	
21	25.00	44.00	170.00	36.38	4.40	10.0	
22	20.00	58.00	300.00	51.00	7.62	17.3	
23	10.00	36.30	200.00	32.40	7.00	12.0	
24	25.00	90.75	250.00	96.25	3.90 +5.50	10.7	
25	30.00	21.60	114.00	19.59	2.01	+6.6	
26	35.00	30.63	155.00	31.11	+0.48	9.3	
27	30.00	29.70	100.00	26.71	2.99	+1.5	
28 29	20.00	10.01	125.00	9.46	0.55	10.0	
30	50.00	44.75	95.00	40.61	4.14	5.4	
30	50.00	44.75	98.00	38.47	6.25	9.2 14.0	

TABLE II b

Absorption of Bilirubin from Sterile Bile in Intestinal Loops
3 Hours Duration

Experiment No.	Amount in		Amount out		Loss	
1 2 3	50.00 30.00 40.00	49 00 52.20 56 00	144.00 148.00 125.00	46.51 45.37 60.04	7.1. 3.49 6.83 +4.04	fer cent 7.1 13.0 +7.2

Experiment No.	Amount in		Amount out		Loss	
1 2 3 4 5	50.00 50.00 50.00 40.00 40.00	mg. 44.00 44.00 44.00 30.20	420.00 90.00 150.00 395.00 107.00	mg. 40.99 47.93 45.00 32.19 31.03	3.01 +3.93 +1.00 +1.99 +0.83	6.8 +8.9 +2.2 +6.6 +2.7

TABLE 11 c

Absorption of Bilirubin from Sterile Bile in Intestinal Loops
5 Hours Duration

experiments did the loss or gain in the pigment exceed the experimental error of the method. In a similar manner the secretion of the entire small intestine was used instead of that of the loop. Again quantitative recoveries were possible.

Fate of Bilirubin in Sterile Bile When Placed in the Intact Small Intestine.—As explained under methods, sterile bile was introduced into the duodenum of an unanesthetized dog by means of the common duct catheter. This bile remained in the small intestine for 1 hour. The animal was then sacrificed and the entire small intestine removed. The stomach was also removed and the contents tested qualitatively for any regurgitated bile. If a positive test for pigment was obtained the experiment was discarded. No bile was ever found in the large bowel if the animal was sacrificed 1 hour after the bile was introduced into the duodenum.

In twelve experiments the loss of bilirubin varied from 24.2 per cent to 64.5 per cent. The mean loss of bilirubin for this series was 43.7 per cent. In all experiments the amount of fluid removed was always less than the amount introduced (Table I).

Fate of Bilirubin in Sterile Bile When Placed in Intestinal Loops.—
Sterile bile did not show any marked bilirubin loss at the end of 2 hours after it had been placed in the intestinal loop. In the majority of instances the bilirubin lost did not exceed the experimental error (Table II). It was thought that perhaps sufficient time was not allowed for the absorption of pigment in the intestinal loop. Therefore, eight other experiments were done, this time allowing the bile in three instances to remain in the loop for 3 hours and in five experiments

for 5 hours. It can be seen that in the majority of instances the loss of bile pigment did not vary markedly from the error of the method.

Fate of Bilirubin in Contaminated Bile When Placed in Intestinal Loops.—In a similar manner contaminated bile was placed in the

TABLE III

Absorption of Bilirubin from Contaminated Bile in Intestinal Loops
2 Hours Duration

Experiment No.			Amou	at out	Loss		
	cc.	mg.	cc.	ms.	rig.	per cens	
1	20.00	12.00	222.00	10.88	1.12	9.3	
2	20.00	12.00	95.00	11.40	0.60	5.0	
3	25.00	12.00	125.00	10.75	1.25	10.4	
4	18.00	10.80	160.00	10.40	0.40	3.7	
5	18.00	10.80	90.00	10.75	0.05	0.4	
6	18.00	10.80	89.00	8.90	1.90	15.3	
7	60.00	52.68	200.00	50.63	2.05	3.7	
8	25.00	28.50	218.00	30.74	+2.24	+8.5	
9	25.00	28.50	89.00	28.30	0.20	0.7	
10*	35.00	61.60	174.00	60.39	1.21	1.9	

<sup>\* 3</sup> hours.

TABLE IV

Absorption of Bilirubin from Infected Bile in Intestinal Loops
1 Hour's Duration

Experiment No.	Amou	nt in	Amou	ot out	Loss		
	cc.	mg.	cc.	mç.	rig.	fer cent	
1	30.00	14.40	83.00	13.45	0.95	6.6	
2	30.00	14.40	68.00	12.78	1.62	11.2	
3	30.00	14 40	125.00	13.50	0.90	6.2	
4	30.00	10.65	75.00	9.50	1.15	10.7	
5	30.00	10.65	50.00	9.00	1.65	15.4	
6	30 00	8.47	65.00	7.45	0.93	10.8	
7	30 00	8 47	95.00	8 36	0 11	1.3	
Average.		· · · · · · · · · · · · ·		• • • • • • • • • • • • •		8.8	

intestinal loop. The organisms found in this bile were usually the streptococcus and staphylococcus. In nine experiments the bile was allowed to remain in the loop for a period of 2 hours and in one

experiment for 3 hours. In only one instance did the recovery show a loss greater than the limit of the experimental error (Table III).

Fate of Bilirubin in Infected Bile When Placed in Intestinal Loops.— The bile used for these experiments was grossly infected with colon organisms. Also, the liver of the animals secreting this bile was damaged by the previous injection of a solution of colon bacilli into the biliary tract. Often the bile collected from these animals had an orange color and a putrid odor. When this was mixed with the acidalcohol solution instead of obtaining the characteristic color changes which bilirubin normally undergoes, they were changed immediately to a deep red color. It was, therefore, usually impossible to estimate the bile pigment of the bile by this method. However, it was sometimes possible to obtain bile from these animals which was not so markedly affected and thus bile pigment could be determined. Using this type of bile seven experiments were carried out, the bile remaining in the loop for 1 hour. In three of these the amount lost was within the experimental error of the method while in another three the loss was very close to the limit of error (Table IV).

#### DISCUSSION

The results of these experiments indicate that there is a loss of bilirubin from sterile bile when the entire small intestine is utilized for the experiments. The question arises as to whether the bilirubin has been absorbed, or whether it has been changed to some substance in the lumen of the intestine that can no longer be estimated as bilirubin. Since it was found that complete recovery of bilirubin was possible when bile was incubated at body temperature with jejunal loop secretions for 2 hours, it can be concluded that such juice has no effect upon the bile pigment even after 2 hours contact. This was also true for juice from the entire small intestine. It is a fact, however, that urobilin is formed in the intact intestine.

The question naturally arises as to whether the pigment is absorbed as bilirubin or is converted to urobilin to be absorbed or excreted. The data presented cannot answer this question. McMaster and Elman (2) were able to show that urobilin is absorbed from the intestine. They found an increase in pigment output through bile fistulae after the introduction of urobilin into the intestine. Although

Blankenhorn (9) showed that after introduction of bile into the small intestine, there was little or no increase of bilirubin content of the blood in the portal vein as compared to that of the jugular vein, there was a marked increase of urobilin in blood of the portal vein. There was also an increase of urobilin in the lymph of the lymphatic vessels which led from the intestine. The experiments of Blankenhorn strongly suggest that most of the bile pigment absorbed from the intestine is carried away as urobilin. A consideration of the work of Blankenhorn and this present study would indicate that the intestinal contents and mucosa are capable of changing bilirubin to urobilin. Bollman, Sheard and Mann (10) were unable to find any increase in bilirubin content in the blood of the mesenteric veins after the introduction of bile into the intestine. These investigators, however, did not estimate the urobilin content of the blood.

The results obtained when either sterile, contaminated or grossly infected bile was placed in the modified Thiry loops strongly indicates that under the conditions of the experiments little or no pigment was absorbed. The difference between the loop experiments and those in which the intestinal tract was utilized as a physiological unit emphasizes the care which must be exercised in making generalizations from data obtained from only one portion of a physiological unit.

#### SUMMARY AND CONCLUSIONS

Since there was no loss of bilirubin from the jejunal loop, and no loss of bilirubin when pigment was incubated with juice from the loop segment, or juice from the entire small intestine, it may be concluded that the intestinal juice per se has no effect in converting bilirubin to urobilin in a 2 hour period, and that in the jejunal loop there was no absorption of pigment or no conversion to urobilin. The experiments showing loss of pigment in the entire intestinal tract suggest that in some place other than the jejunal portion of the intestine the combined activity of intestinal contents and intestinal cells does affect the bilirubin in the intestine. Whether the loss of bile pigment under such circumstances is due entirely to conversion, or to conversion and absorption, or to absorption of bilirubin as such, remains to be answered by subsequent investigations.

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# TRANSPLANTATION OF TOOTH GERM ELEMENTS AND THE EXPERIMENTAL HETEROTOPIC FORMATION OF DENTIN AND ENAMEL\*

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Plates 9 to 12

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The histologic proximity of epithelium to such superficial appendages of the organism as the scales, shell, hair, nails and the developing tooth has been discovered within the last century. Moreover, experiments in recent years (2-4) have shown that the epithelium of certain organs in mammals, notably the gall bladder and the urinary tract distal to the kidney, when transposed to certain connective tissue areas regularly causes the formation of osteoblasts and bone in these Since the relationship of the calcified elements of the tooth to epithelium in the developmental stage is strikingly similar to bone forming after epithelial transplantation, and since the inorganic crystallites of teeth and bone are chemically identical even to X-ray diffraction studies (5, 6), it was considered advisable to investigate the odontogenic properties of tooth germ elements in an attempt to induce the extra-oral formation of dentin and enamel. The relatively common heterotopic occurrence of teeth in pathological situations, as in the pituitary (7) and elsewhere, especially in the ovary, as a result of teratomatous tumor formation lent support to the conception that this could be accomplished experimentally.

The idea of the ontogenetic relationship of epithelium to the developing tooth arose mostly as a result of the anatomical studies of Kölliker. Hertwig (8) de-

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scribed the penetration of the jaws by an epithelial sheath, which he believed was essential to tooth formation. The discovery of Tomes (9) that the epithelial enamel organ was present in the armadillo, whose peg-like teeth consist solely of dentin devoid of enamel, and the studies of von Brunn (10, 11), who showed that the entire tooth of rodents is surrounded at some time by the enamel organ, although the dorsal surface of the tooth is composed of dentin and only the ventral surface is covered by enamel, led to the belief that epithelium was necessary in some way for the development of both enamel and dentin. To quote von Brunn: "where there is no epithelial sheath, there can be no odontoblast formation and consequently no dentin," (11).

In the present experiments the technique consisted in the autogenous transplantation of tooth germ elements to the connective tissues of the abdominal wall of young pups; so far as we could discover, this method has not been previously reported. Legros and Magitot (12), however, studied transplanted tooth germ elements in the much more difficult Their results field of homogenous and heterogenous grafting. achieved in the pre-aseptic era are noteworthy. These investigators removed dental follicles from young pups immediately after death and transplanted them subcutaneously into guinea pigs or older dogs. The heterogenous grafts were all lost through suppuration or resorption as were most of the homogenous grafts. The positive results obtained were as follows: 7 of 26 homogenous grafts of the complete dental follicle survived and led to regular development, and in 3 of 16 cases in which the pulp was removed and transplanted alone, the tissue survived and reproduced a new cap of dentin.

#### Methods

Young dogs between the ages of 3 and 6 weeks were used and all operations were done under ether anesthesia. An aseptic manner of operating in the mouth was impossible to attain and was found not necessary; the grafts were extraordinarily free from infection. The instruments used in the mouth were sterilized by heat, the operative field was swabbed with iodine and after isolation of the dental follicle, the tissues were removed with fresh instruments into sterile Ringer's solution. The abdominal operation was then done with asepsis.

The material grafted consisted in the soft tissues of the unerupted permanent canine tooth which was selected because of its large size and ease of accessibility. The canine tooth in the upper jaw was always found anterior and superior to the root of its deciduous precursor; to remove this tooth a 2.5 cm. incision was made

<sup>&</sup>lt;sup>1</sup> Von Brunn (11), page 145.

in the gingiva covering the lateral aspects of the superior maxilla parallel to the root of the temporary canine tooth, down to the bone; the periosteum was reflected and the thin plate of bone overlying the tooth removed with a chisel. With care the tooth and its membranes were removed intact. Removal of the tooth in the mandible was slightly more difficult; after making parallel incisions in the gum margin, medial and lateral to the temporary teeth, the periosteum was reflected from the mandible, and a wedge-shaped piece of bone was sawed away just posterior to the deciduous canine, exposing the permanent tooth in the medial aspect of the mandible. Wounds in the jaw were not sutured.

The dental follicles were opened and the calcified cone of dentin and enamel was always discarded. In a number of cases, an X-ray photograph of the soft tissue was made before transplantation, and in all cases some tissue was excised from the graft for histological control. It was regularly possible to cleanly remove the pulp with its odontoblast layer from the dentin without adherent calcified particles, but it was much more difficult to dissociate calcified enamel from the ameloblast layer, and in many cases a few small bundles of preformed enamel were transplanted inadvertently with the epithelial layer.

The abdominal site for reception of the transplant was prepared by making a 2 cm. incision in the skin and external oblique muscle. The grafts were inserted between the internal and external oblique muscles, and the latter was then sutured as well as the skin with fine silk.

The animals were kept from 2 to 56 days on a stock diet of meat scraps, bread and lettuce, with an unlimited supply of cow's milk. A special group in which the known vitamin content of the food was maintained at a high level was fed in addition by daily gavage a supplementary ration consisting of butter 12 gm., tomato juice 12 cc., fresh yeast 4 gm., irradiated ergosterol (1000 D) 2 drops.

The soft tissues of the developing tooth at this stage consist of (a) the pale white pulp with odontoblasts on the surface and (b) the red epithelial layer consisting of an innermost cylindrical cell layer, the ameloblasts; next a cuboidal row of cells, the stratum intermedium; and outermost a thin layer of flattened epithelium surrounded by connective tissue with a rich vascular supply. odontoblast-pulp membrane is joined at its base, where the blood vessels and nerves enter, with the more embryonic portion of the epithelial layer, the epithelial sheath of Hertwig, precursor of the tooth root. The membranes are separated normally by the more or less calcified tooth shell of dentin and enamel which in these experiments was always removed and discarded. The relationship of these membranes in the graft was, as will be shown, a determining factor in the product of the transplantation. The membranes were transplanted in various combinations and the distinct difference in color made for the ease of anatomical separation. First, the membranes were cut across so that the epithelial layer (Method A) and the pulp-odontoblast layer (Method B) could be transplanted to different parts of the abdominal wall. Secondly, the membranes were transplanted together (Method C) so that the epithelial layer had a quasi-normal relationship to the

pulp and the cylindrical ameloblasts were in close proximity to the odontoblasts; and in another series the epithelial layer instead of ensheathing the pulp was drawn upwards so that these membranes were in continuity but not approximated (Method D). Thus in the experiments done using Method D the epithelial layer bore a "skin-the-rabbit" relationship to the pulp. In another series (Method E) a transverse section of the pulp was circumcised so that all of the odontoblasts were removed together with a thin layer of underlying pulp cells; thus the surface cells of the pulp were transplanted to a different location from that of the central pulp cells. In Method F, a sheet of mouth epithelium covering the mandible was excised and transplanted to the abdominal wall.

#### RESULTS

Since great variations resulted depending on the nature and relationships of the material used for transplantation, the 6 series of experiments will be separately described. The infrequency of inflammation around the grafts, was noteworthy in view of the impossibility of maintaining complete asepsis in securing the grafts.

## Method A. Isolation of the Epithelial Layer and Transplantation to the Abdominal Wall

Twenty-one experiments were done in this group; an animal was killed every 2nd day from 2 to 30 days after transplantation. It was somewhat difficult to remove the epithelium from the enamel without obtaining a few adherent enamel bundles. The preformed enamel bundles usually were small and regular, and the enamel rods relatively long and in microscopic section had a straight regular edge making it easy to differentiate transplanted from newly formed enamel.

These enamel bundles did not excite a specific response in the tissues; giant cells were completely lacking around them although present in great numbers around fortuitously adjacent silk fibres; the bundles uniformly became encapsulated by fibrous tissue or epithelium and the enamel rods appeared to be histogenetically inert.

The most significant features of the experiment were the total disappearance of the cylindrical ameloblasts, the absence of new enamel formation and failure of the epithelium to form cysts.

The epithelium survived only as a stratified epithelium composed in part of squamous cells and in part basal cells. As early as the 2nd day after transplantation, the cylindrical character of the ameloblasts was lost and the epithelium was forming islands and cords of cells which persisted. In the later stages, it was seen that some of the islands were composed entirely of basal cells, and others almost exclusively of stratified squamous cells. At 4 days, expansion of the epithelium

was easily observed, already more extensive than it becomes normally in the jaws. Mitotic figures were numerous. In later stages epithelial pearl formation was not unusual. It was of interest to observe the relationship of the basal to the squamous cells; in the largest islands, composed chiefly of basal cells, the center sometimes contained squamous cells, as if the basal form was dependent on a more favorable vascular relationship. Some of the epithelial islands surrounded connective tissue, so that in the plane of the section, the fibroblasts were completely encompassed.

There was no new formation of enamel detectable in any of the sections.

The epithelium showed no tendency to form cysts, except the small obviously necrotic centers of some large squamous cell islands, containing epithelial cells, whose nuclei almost filled them. This is an exceedingly unusual happening in epithelium under these conditions of subcutaneous transplantation.

### Method B. Transplantation of the Isolated Odontoblast-Pulp Layer

As compared with the epithelial layer, it was easy to regularly remove the pulp with its surrounding odontoblasts from the dentin cap without any adherent traces of calcified material; this was repeatedly demonstrated by X-ray examination as well as by biopsy of random areas before transplantation, for histological examination. Twenty-four experiments were done in which this material was transported to the connective tissue areas of the abdominal wall or thigh. The results were similar, but the ease of identification of the tissue in the abdomen, as well as the absence of calcified elements in this site normally, made this location more satisfactory for the purposes of the experiment.

The duration of the experiment was from 1 to 26 days. In 12 of the experiments, exclusively those between 14 and 26 days, small masses of calcified dentin were found. Further proof that the dentin found in the later experiments was newly formed in the heterotopic location occurred in the observation that no dentin was found in any of the experiments before the 14th day.

In the material recovered for examination between the 2nd and 10th days, it was impossible to recognize the odontoblasts as such, but after this time aggregation of more densely staining compact cells associated with an eosinophilic protein secretion identified the future site of dentin formation. The pale staining stellate pulp cells with the included surviving empty preformed capillaries easily differentiated the transplant from the native abdominal connective tissue.

The calcified dentin occurred roughly at the junction of the pulp and the encapsulating fibroblasts. The dentin was found in circular, or irregular plaque form with collections of odontoblasts located almost exclusively on one surface and the other surfaces were encapsulated by common connective tissue. The

circular masses of dentin in places appeared just within the interior of the pulp. They were always surrounded by odontoblasts, usually both on the surface and in the interior. These masses of dentin had well defined canaliculi, resembling the tubules of normal dentin. By the 15th day the dentin was always calcified, but as late as the 26th day there was always an uncalcified zone separating it from the odontoblasts.

Some of the dentin enclosed living cells as if it had been secreted around fibroblasts which were thus included; in other places the interstitial cells closely resembled bone corpuscles. There was observed every gradation between true dentin, through osteodentin to true bone. As studies of the teeth in vitamin A and C deficiency (14, 15) have established, there is a close relationship between the cells known as odontoblasts and osteoblasts; and these observations were fully confirmed by the present experiment.

## Method C. Transplantation of Odontoblast-Pulp and Epithelial Layers So That a Quasi-Normal Relationship Obtained

This experiment was done 6 times. The results were identical in each case although the duration of growth varied from 21 to 31 days after transplantation. The interesting features observed were the preservation of the cylindrical character of the ameloblasts and the formation of new enamel in normal relationship to these cells and to newly formed dentin.

The ameloblast and odontoblast layers survived the transplantation and produced a relatively large amount of the calcified tooth substances, enamel adjacent to the ameloblasts, and dentin close to the odontoblasts. Relatively long stretches of enamel epithelium and of enamel were found; the outline of the row of enamel rods was irregular and wavy, the enamel rods were short, and thus there were distinct differences between the newly formed enamel and the irregular narrow but long bundles with straight edges which characterized preformed transplanted enamel. Moreover enamel formed before the transplantation had no regular relationship to dentin such as existed here. In each case Hertwig's sheath was found, showing that this still undeveloped embryonal tissue is capable of survival in a new location. The growth of the epithelial and odontoblast layers was not entirely regular, in places these layers invaginated the pulp so that a shelf-like arrangement of dentin and enamel appeared in the pulp. The growth of the cell layers was not perfectly symmetrical, so that in some areas either layer was lacking. In the regions where ameloblasts were missing, the odontoblasts proliferated to form large irregular masses of dentin and small spheroidal denticles.

absence of the odontoblast layer, however, the cylindrical ameloblasts did not persist and were replaced by stratified squamous degeneration of the epithelium without formation of enamel rods. Thus enamel was only deposited on dentin, although dentin formation was definitely independent of enamel production, and the enamel rods formed only in association with cylindrical cells.

### Method D. Transplantation of Epithelial and Odontoblast-Pulp Layers Together without Preservation of Normal Proximal Relationship of the Membranes

Twelve experiments were done using these membranes attached at the base of the follicle but with a deliberate attempt to avoid contact of the cylindrical ameloblasts with odontoblasts. The duration of the experiments varied from 9 to 56 days. The results can be interpreted as a combination of the results obtained by use of Methods A and B. At 9 days there was found early uncalcified dentin in the region of the pulp and masses of basal and squamous cell epithelium away from it. In 2 of the experiments the epithelial sheath of Hertwig was identified but cylindrical ameloblasts were not observed and there was no evidence of newly formed enamel. The only observable difference in the dentin formation was an apparent increase in the amount of dentin formed. The largest amount of dentin including cellular dentin and bone observed in these experiments occurred in these sections. As mentioned under Method A, in transplantation of the epithelial layer a few bundles of preformed enamel usually adhere to the epithelium, and in the present experiment not infrequently there were found masses of these preformed enamel rods entirely encapsulated by dentin. This is, of course, proof of the new formation of dentin in the abdominal wall. In certain areas, here as in Method B, there were observed irregular serpiginously contorted masses of dentin formed presumably as a result of the opportunity of the pulp to undergo free growth for a limited period.

# Method E. Transplantation of the Center of Pulp to the Abdominal Wall as Compared with the Peripheral Portion Containing the Odontoblasts

In this experiment, the center of the pulp was completely freed from the odontoblasts, by a circumcision of the periphery. A 4 mm. transverse section was cut equatorially through the pulp, which was then pinned to a block of wood with needles, allowing the surface layer of odontoblasts to be easily and completely excised with a razor. Each of the 2 masses of tissue consisting of the center and periphery of the pulp was transplanted to separate portions of the abdominal wall.

In 5 experiments with the center of the pulp, the tissue was recovered at 20, 24 and 29 days; the stellate pulp cells were easily seen, but there was no trace of dentin or calcified material found.

In 5 similar experiments with the surface layer of odontoblasts and necessarily the immediately subjacent pulp cells, the duration of the experiment being the same, small islands of dentin, with odontoblasts arranged along one surface were found in 4; in the 5th experiment, terminated at 29 days, no calcified tissue could be identified.

## Method F. Transplantation of Gingival Epithelium to the Abdominal Wall

The gum was painted with iodine, in 7 experiments, and a thin layer of epithelium covering the lateral aspect of the mandible excised and transplanted to the abdominal wall. The graft survived 32, 47 and 69 days. In all cases there was infection, and in 2 experiments no epithelium could be identified at the termination of the experiment.

In brief, the epithelium survived, proliferated to form a cyst lined with squamous epithelium and produced no calcified dental substance. The basal papillae of the original epithelium survived, but the newly formed portion of the cyst did not reproduce this papillary pattern but persisted in the form of a flat stratified squamous cell layer. A leucocytic invasion surrounded the epithelium in all cases.

#### DISCUSSION

Aside from the actual heterotopic formation of dentin and enamel the most interesting feature of these experiments was the epithelial cytomorphosis. It seems clear from the histological evidence that when the enamel epithelium is associated in an approximately normal relationship to the odontoblasts and pulp, the cylindrical character of the ameloblasts is preserved with the production of new enamel rods and that otherwise these properties are lost. The maintenance of the form of the epithelium and its function in producing enamel seems to be due to the influence of the mesodermic tissue on the epithelial

ameloblasts themselves, and if so this is a converse situation to the effect of epithelium such as that lining the lower urinary tract on 207 certain strains of fibroblasts causing them to change form and function in the production of bone. This mutual interrelationship of supporting tissues and epithelium on each other in the postnatal state, whereby changes in the one or the other are reflected in pronounced anatomical and physiological variations in the tissue, is of great interest and is being further studied in this laboratory.

The inability of the epithelium to form cysts, excluding the pseudocysts in large degenerating squamous islands, is likewise a most remarkable property of transplanted epithelium and in this respect this epithelium is unique in our experience with normal epithelia. absence of cyst formation in this stratified baso-squamous epithelium at once calls to mind the islands of epithelium normally occurring in the periodontal membrane of all animals after the tooth has erupted above the gingival margin. This epithelium first described by Malassez (13) likewise shows no tendency to cyst formation under normal conditions and the appearance of the enamel organ when its normal relationship to the pulp is lost through transplanting is quite similar

It may be of interest to return briefly to our original thesis and to discuss the influence of epithelium on the formation of calcified tooth elements and to the similarity of this process to the ossification-inducing action of the epithelium as above mentioned. It is certain that for every developing tooth, there is in the closest relationship an epithelial downgrowth from the oral cavity. The hypothesis of von Brunn, since accepted by many investigators, was that no calcified tooth elements can be built without this epithelial ingrowth, and that epithelium stimulates the maxillary connective tissues to form odontoblasts and thus dentin. There thus appears a similarity to the osteogenic influence of bone-stimulating epithelia on fibroblasts. The Dresent experiments, however, indicate that the view of von Brunn puoted above that "where there is no epithelial sheath, there can be o odontoblast formation and consequently no dentin" needs modificaon, in that odontoblasts, which have arisen as a result of epithelial fluence on connective tissue, do not further need for their function e presence of epithelium. The evidence shows that the odontoblasts

arose as a result of the action of epithelium on the maxillary mesenchyme, causing a permanent change in these cells. These transplanted odontoblasts then were capable of forming dentin in the abdominal wall without further aid from the epithelium. The epithelial influence on the fibroblasts thus has a local hereditary influence as far as these cells are concerned, both in their form and function, which is precisely what occurs with the osteogenic epithelia. Unpublished experiments have clearly shown in this laboratory that the boneforming properties induced in fibroblasts by epithelial stimulation are thereafter inherited; bone formed from transplantation of urinary bladder epithelium to the abdominal wall was later freed from the epithelium, was fractured and promptly the break healed by bony union, through the inheritance of the osteogenic character in the daughter cells of the original fibroblasts that had formed bone, although the original epithelial osteogenic stimulus had been removed.

An obvious difference between the present experiments and the osteogenic stimulus of epithelium on fibroblasts, in that enamel epithelium did not stimulate the abdominal wall fibroblasts to form calcified elements, may perhaps be best explained by the fact that in the absence of the mesodermic pulp elements, the enamel organ loses its specialized form and degenerates in the direction of gingival epithelium.

It is hoped that the development of a method whereby the calcified components of the tooth are formed in a bacteriologically sterile site such as the abdominal wall will provide new means for study both of kataplastic process such as dental decay under aseptic conditions in the absence of saliva, as well as the histogenic details and physiology of the early formative stages of the calcified tooth elements.

#### SUMMARY

The formation of dentin and enamel in the abdominal wall in young pups was achieved by transplantation of the soft tissues of the developing tooth germ. An interesting finding was the cytomorphosis of the epithelium of the enamel organ. When this was transplanted so that the ameloblasts were in contact with the odontoblasts the cylindrical character of the epithelial cells was preserved and enamel was produced; otherwise the cylindrical shape of these cells was lost and a stratified epithelium resulted, resembling the gingival and certain

tumors (the adamantinoma) of the jaw and related structures. degenerated epithelium did not produce enamel and had an important characteristic of not forming cysts in a closed connective tissue space, instead forming islands and cords of cells with epithelial pearl formation. Thus the influence of mesodermic connective tissue derivatives on the form and function of epithelium is presented. The odontoblasts were found capable of survival as such and readily formed new dentin in transplantation; the stellate cells of the pulp were inert from the standpoint of inducing calcification.

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## EXPLANATION OF PLATES

The tissue from which these photomicrographs were made was prepared by formalin fixation, dehydration and paraffin impregnation. The tissue shown in Figs. 6 to 12, in addition, was decalcified in 5 per cent nitric acid. All sections were stained with hematoxylin and eosin.

Fig. 1. Transplantation of enamel epithelium to abdominal wall (Method A), 4 days. The cylindrical character of the ameloblasts is now represented only by spindle-shaped cells; most of the epithelium has assumed a stratified squamous

- Fig. 2. The same as Fig. 1, 17 days. The enamel epithelium has survived as islands formed chiefly of basal cells. ×160.
- Fig. 3. The same as Fig. 1, 21 days. Two islands of epithelium (A, A') are shown, with an epithelial cord at B.  $\times 145$ .
- Fig. 4. The same as Fig. 1, 16 days. A large epithelial island with many squamous cells is shown. ×115.

#### PLATE 10

- Fig. 5. Transplantation of odontoblast-pulp membrane to abdominal wall (Method B), 14 days. At the junction of the pulp (P) with the abdominal connective tissue cells (F) there is an island of early dentin, only slightly calcified; odontoblasts may be seen at O.  $\times 145$ .
- Fig. 6. The same as Fig. 5, 24 days. At the interface between pulp (P) and connective tissue (F), a large island of dentin has formed; odontoblasts (O) are seen on one surface.  $\times 140$ .
- Fig. 7. The same as Fig. 5, 21 days. A serpiginous mass of dentin has formed at the junction of the pulp (P) and the encapsulating fibroblasts (F).  $\times 145$ .
- Fig. 8. The same as Fig. 5, 28 days. Five oval and circular masses of dentin, and a plaque form are seen at the junction of pulp (P) and connective tissue (F). Note the odontoblasts on the surface of the denticles.  $\times 115$ .

#### PLATE 11

- Fig. 9. Transplantation of epithelial and pulp odontoblast layers together, but not in contact (Method D), 28 days. A large mass of slightly cellular dentin has formed.  $\times 215$ .
- Fig. 10. Transplantation of epithelial and mesodermic layers together, with the ameloblasts in intimate contact with the odontoblasts (Method C), 20 days. Note Hertwig's sheath (H), newly formed dentin and enamel (E), squamous degeneration of epithelium (D).  $\times 26$ .

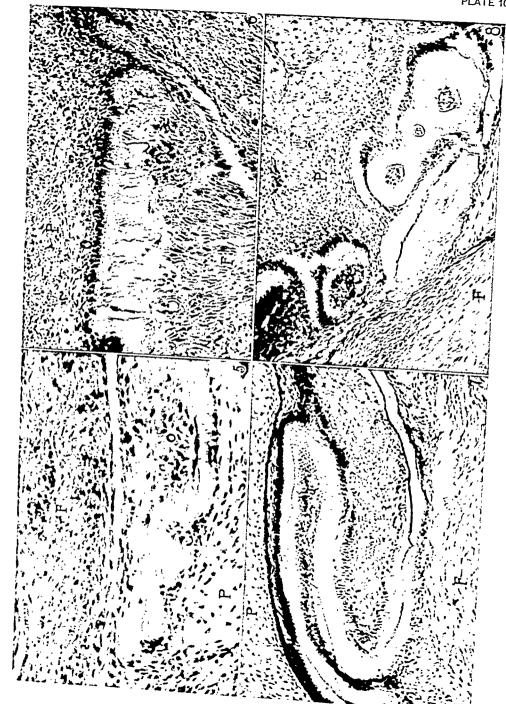
#### PLATE 12

- Fig. 11. The same as Fig. 10, 21 days. The pulp (P) with its odontoblasts (O) may be seen separated by a new formation of dentin and enamel from the cylindrical ameloblasts (A). The surrounding connective tissue of the abdominal wall is seen at F.  $\times 370$ .
- Fig. 12. The same as Fig. 10, 26 days. A somewhat tangential section of dentin (D) and enamel (E) to show the hexagonal enamel prisms. The ameloblast layer is seen at A.  $\times 405$ .



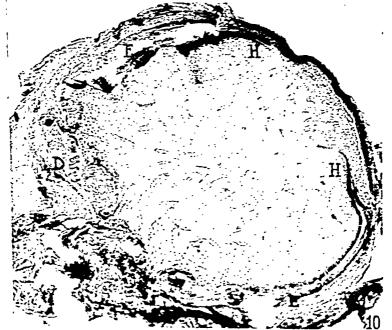
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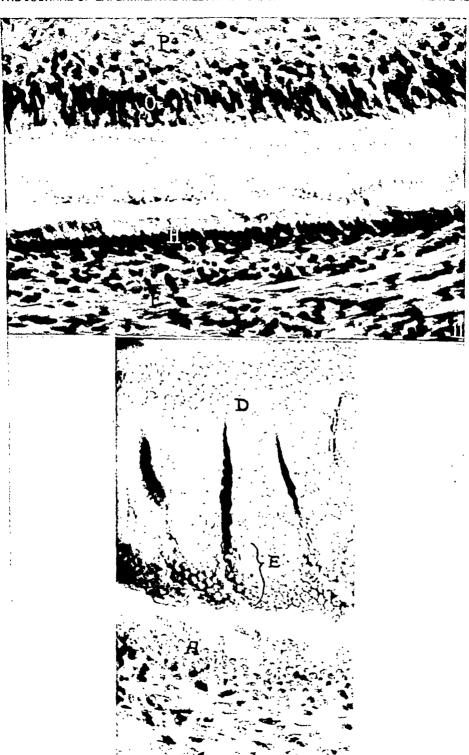






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## VACCINATION OF MONKEYS AND LABORATORY WORKERS AGAINST PSITTACOSIS

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Cases of psittacosis are still occurring in birds and in human beings, and it is obvious that the disease has become endemic (1) in the breeding aviaries of this country and that cases among human beings will continue to occur until the breeders close the aviaries or get rid of their infected stock. In view of the fact that laboratory workers exposed to infection frequently develop psittacosis it seemed advisable to ascertain whether individuals who have had the malady are refractory to reinfection and to devise a method for the vaccination of human beings against the disease.

#### EXPERIMENTAL

In approaching the problem of vaccination of laboratory workers against psittacosis we decided that it would be advisable to ascertain what could be accomplished with monkeys before anything was attempted with human beings. Consequently, we set out to determine (1) whether monkeys that have recovered from psittacosis pneumonia are refractory to reinfection, (2) whether such monkeys have neutralizing antibodies in their sera, (3) whether large amounts of psittacosis virus administered to monkeys intramuscularly and intravenously produce a serious infection, e.g. psittacosis pneumonia, and (4) whether monkeys that have received repeated small doses of active psittacosis virus intramuscularly, that is vaccinated monkeys, are refractory to intratracheal inoculations of active virus and possess neutralizing antibodies in their sera.

Resistance of Monkeys to Reinfection by Psittacosis Virus

Previous work (2-1) has evidenced the fact that parrots, rabbits, and mice that have recovered from psittacosis possess a heightened

resistance to reinfection. It has also been found possible to produce a psittacosis pneumonia in monkeys (5) by means of intratracheal injections of active virus. When the present investigation was begun, however, there was no definite evidence available regarding the state of resistance of recovered monkeys to psittacosis virus. Before the vaccination of monkeys was undertaken, it seemed wise to determine whether monkeys that have recovered from psittacosis pneumonia are more refractory to the virus introduced intratracheally than are normal control animals. The desired information was obtained in the following manner.

In each of Monkeys<sup>1</sup> 19, 20, and 22 (see Table I), a psittacosis pneumonia was produced by means of an intratracheal injection<sup>2</sup> of 2 cc. of a 1 per cent emulsion in Locke's solution of livers and spleens from mice infected with psittacosis virus. The animals became sick and X-ray pictures of their lungs revealed extensive consolidation. 43 days after the infection in Monkeys 19 and 20 and 105 days after the infection in Monkey 22, each of Monkeys 19, 20, 22, and 28 (normal control animal) received intratracheally 2 cc. of a 1 per cent virus emulsion similar to that described above. Monkey 22 was not sick and X-ray photographs of the lungs showed no consolidation. Monkeys 19 and 20 were not sick, but X-ray pictures showed a slight involvement of their lungs. Monkey 28 (the control), however, was sick and refused to eat, and X-ray photographs of its lungs revealed considerable pneumonia around the hilum and at both bases. Following the X-ray examinations on the 7th day after inoculation the 4 monkeys were sacrificed. The lungs of Monkey 22 were essentially normal macroscopically and microscopically. In the gross, the lungs of Monkeys 20 and 19 showed respectively a slight amount of pneumonia of both lower lobes and a few scattered areas of consolidation of the right upper and lower lobes. Stained sections of the consolidated tissues revealed some thickening of the alveolar walls and mononuclear elements in the alveolar spaces. No hemorrhages or areas of necrosis were seen. In Monkey 28 (normal control) practically all of the right lower lobe, half of the left lower lobe, and approximately a quarter of the right upper lobe were consolidated. Stained sections of these lobes showed hemorrhages, areas of necrosis, thickening of the alveolar walls, desquamation of the alveolar epithelium, and mononuclear exudate with fibrin in the alveolar spaces. The gross and microscopic pictures in the lungs of the control Monkey 28 were decidedly different from those found in the lungs of the reinoculated animals, Monkeys 19, 20, and 22. The results of the experiment are summarized in Table I.

<sup>&</sup>lt;sup>1</sup> Approximately half-grown *Macacus rhesus* monkeys were used in all experiments.

<sup>&</sup>lt;sup>2</sup> All operations were performed under light ether anesthesia.

TABLE I

Summary of Experiments Dealing with Immunization of Monkeys against Psittacosis

	Primary inject	tions	=====		Intr	Seco: atrach	ndary al tes	injecti t for ir	on nmunity	
-	1	R	ezction		pri-	E	videno	e of in	amunity	
	-				nster				Neutra tion to	liza- est
Monkey No.	Method of injection		Temperature	X-ray	Length of time after pri- mary injection	Clinical	Temperature	X-rny	Length of time after primary injection	Result
1 2 3			- - ±	- - -	days 47 47 73	++++	++++	++++	<i>∂ay</i> s 201*	+
4 5 6	3 weekly injections in- tramuscularly	- - -	± - -	_	73 43 57	+++++	-	+++++	} 41	+
7 8		-	-	_	62 142	  -   ±	+++++++++++++++++++++++++++++++++++++++	+ +	34	+
						<u></u> -	<u> </u>	<u> </u>	139	+
9 10 11 12 13 14	5 weekly injections in- tramuscularly		# # # # -	-	33 59 27 41 48 128	+++++=	++++	++++	25 33 125	+++++
15	1	_	=	-	100	+	≐	-		
17	1	-	-	-	70	+	=	-		
19 20 2: 2: 2:	2 2 3 3	+ + + + +	+ + +	++++	43 43 70 105 190	++++	† - + +		112	+++
2 2	5 1 injection intratra- cheally	(Did	utopsy ·	+)						
2	6 7 8	++	+ -	+					172	=
3	9 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	+++++++	+=	++++					156	+

Serum for this test was obtained 201 days after secondary injection.

† Killed and autopsied.

The experiment just described was repeated and similar results were obtained. From these experiments it is obvious that monkeys that have recovered from psittacosis pneumonia are more refractory to infection by the virus introduced intratracheally than are normal monkeys. From our experience it seems that an increased resistance to psittacosis is acquired slowly. Therefore, one should wait at least a month after the primary inoculation before testing for a heightened resistance in the animals by means of a secondary injection. Moreover, the immunity is only relative, and if too large an amount of virus is administered in the secondary inoculum one will fail to demonstrate a refractory state.

### Neutralizing Antibodies in the Sera of Monkeys Recovered from Psittacosis Pneumonia

In addition to determining the presence of a refractory state in monkeys to reinfection by psittacosis virus we attempted to ascertain whether the sera of such animals possess neutralizing antibodies for the active agent. However, when we undertook to demonstrate the presence of the antibodies, it became evident at once that a test in which only a few mice and one or two dilutions of virus are employed is not adequate. We then devised the following test which seems to give satisfactory results.

A 20 per cent liver and spleen emulsion from infected mice was prepared and centrifuged for 10 minutes at 3000 R.P.M. The supernatant fluid was collected and decimal dilutions of it were made with Locke's solution. These dilutions were not allowed to stand for any length of time because the virus dies very rapidly under such conditions. Definite amounts of at least four of the dilutions of virus were mixed with equal amounts of the sera being tested. A positive and a negative control serum were run in each experiment. The mixtures were incubated at 37°C. for 2 hours. Then 5 mice<sup>3</sup> were inoculated intraperitoneally with each mixture, each mouse receiving 0.5 cc. The mice were observed for 18 days. The percentage of mice that died and the average time of their death were noted. A summary of an experiment is shown in Table II.

An examination of the results shown in Table II reveals that more of the mice receiving mixtures of normal serum and virus died than did those receiving virus mixed with the other sera. Furthermore,

<sup>&</sup>lt;sup>3</sup> The mice must be from a relatively uniform stock as regards susceptibility, and must also be of approximately the same age and weight.

the average time of death for the former set of mice was shorter than that for the latter. The differences in percentages of deaths and in the average times of death are not strikingly significant when considered separately, but when the two factors are considered together

TABLE II

Summary of an Experiment Illustrating the Manner in Which Neutralization Tests

Were Performed

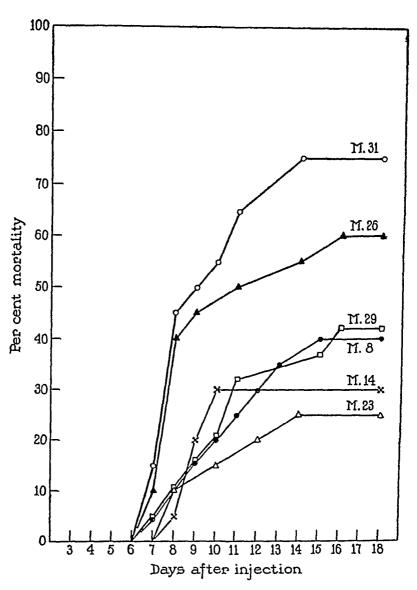
Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of deaths	Average time of death	Neutraliza- tion index			
		Known no	rmal serum	+ virus dilutions					
10 <sup>-4</sup> 10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup>	5 5 5 5	5 5 5		5, 6, 6, 6, 6 5, 6, 6, 6, 7 7, 7, 8, 8, 8 8, 8, 8, 9, 9					
	20	20	100		6.9	14.5			
	Serum being tested + virus dilutions								
10 <sup>-4</sup> 10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup>	5 4 5 5	5 4 5 1		6, 7, 7, 8, 8 6, 7, 8, 9 8, 8, 8, 12, 12 10					
	19	15	79		8.3	9.5			
		Known in	nmune serun	n + virus dilutions					
10-4 10-5 10-6 10-7	5 5 5 4	5 5 4 1		6, 7, 7, 9, 9 5, 8, 10, 10, 10 9, 10, 12, 12 10					
<del></del>	19	15	79		8.9	8.9			

Neutralization index = percentage of deaths divided by average time of death.

the results of the experiment assume considerable significance. In order to express both factors in one figure we have chosen as a neutralization index the percentage mortality divided by the average time of death.

In the manner described the sera from a number of monkeys that

had recovered from psittacosis pneumonia 39 to 187 days previously were tested for the presence of neutralizing antibodies and it was found



TEXT-Fig. 1. Graphic portrayal of the results of a neutralization test on specimens of sera from 1 normal monkey (M. 31) and from 5 monkeys (M. 8, 14, 23, 26, 29) that had recovered from psittacosis pneumonia.

that in the majority of the sera such antibodies were present From experience it has been learned that the antibodies may appear slowly.

Consequently, a month or more was allowed to elapse after infection before the sera were drawn for the neutralization tests. The results of one experiment are portrayed in Text-fig. 1 (M. 31 represents the results obtained with serum from a normal monkey) and show that M. 29, M. 8, M. 14, and M. 23 definitely possessed neutralizing antibodies. It is hard to interpret the results obtained with the serum from M. 26.

# Effect of Psittacosis Virus Administered to Monkeys Intravenously and Intramuscularly

Before undertaking the vaccination of monkeys with active virus we wanted to know whether large amounts of psittacosis virus administered intravenously or intramuscularly would produce a serious infection such as a pneumonia. From previous work (5) we had been led to believe that psittacosis virus introduced into monkeys in such a manner is relatively harmless. Nevertheless, 2 experiments, one of which will be described, were performed in order to obtain definite evidence concerning the matter.

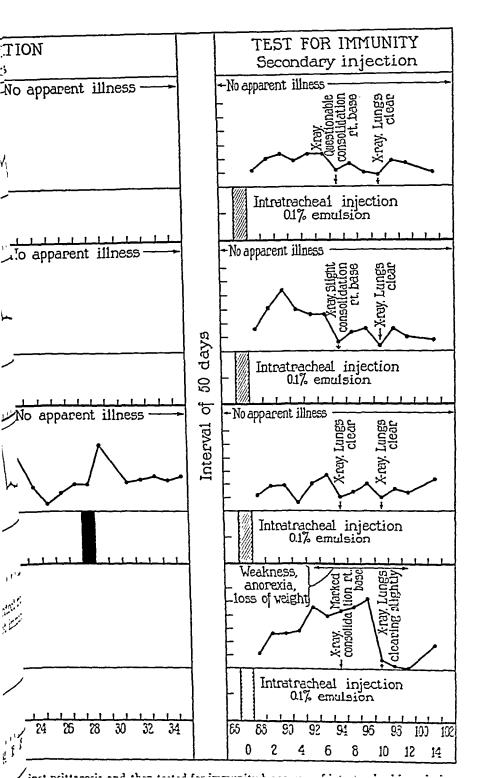
A 1 per cent emulsion of livers and spleens from mice sick of psittacosis was made and centrifuged at 3000 R.P.M. for 5 minutes. The supernatant fluid was removed and used as an inoculum. Monkeys 21 and 25 received respectively 2 cc. of the inoculum intratracheally, while each of Monkeys 17 and 18 received 2 cc. of the inoculum mixed with 3 cc. of Locke's solution intravenously and 0.5 cc. of the inoculum intramuscularly. Monkey 25 became very sick and died 5 days after the inoculation. At necropsy an extensive psittacosis pneumonia was found involving all lobes. Monkey 21 became very sick, had fever, and refused to eat. X-ray photographs of the lungs revealed extensive pneumonia. The animal finally recovered, however. Monkeys 17 and 18 were never sick and had no fever. Furthermore, X-ray pictures of their lungs revealed no evidences of pneumonia.

The results of the experiment described above and of a similar one (Monkeys 15, 16, 19, 20) not detailed are shown in Table I and clearly indicate that large amounts of active psittacosis virus can be administered intravenously and intramuscularly to monkeys in relative safety, while similar amounts of virus given intratracheally cause extensive pneumonia which may be fatal.

### Vaccination of Monkeys against Psittacosis

At this point we believed that we were ready to proceed with the vaccination of monkeys against psittacosis. To this end 6 experi-

PERIOD OF IMMUNICA Primary injections °F. temperature 106 105 104 103 102 101 - 2 Lungs Rectal Monkey Xray emulsion virusorgan Intramuscular injection 10% emulsion . გ 1 1 1 1 1 1 1 1 °F. temperature r. Luhgs Lungs clear 106 105 104 103 102 101 - 2 Rectal 4 Monkey -X ray. virusorgan Intramuscular injection 10% emulsion Cc.of ŧ °F. temperature Lungs clear 106 105 104 103 102 101 2 . Lur g. Clear Rectai Monkey \*Xray. \*X-ray. .¥KĽ: emulsion Cc. of virusorgan Intramuscular injection 1 10% emulsion °F. Control monkey temperature 106 105 104 103 102 101 — 2 Not immunized Rectal な Monkey emulsion virusorgan <del>ن</del> ئ 1 20 18 16 14 2 12 10 0 Days after injection



ments were performed in which 14 monkeys (Nos. 1 to 14 in Table I) were vaccinated and then tested for immunity. Seven normal monkeys (Nos. 22, 24, 26, 27, 29, 30, 31 in Table I) were used as controls in the tests. Inasmuch as all of the experiments were conducted in approximately the same manner only one will be described in detail.

Each of Monkeys 3, 4, and 10 received intramuscularly at weekly intervals 3 doses (0.1 cc., 0.15 cc., 1.0 cc.) of active psittacosis virus contained in a fresh 10 per cent emulsion of the livers and spleens of infected mice. In addition to these inoculations, Monkey 10 received 2 more weekly injections (1.5 cc., 2.0 cc.) of similar emulsions. During the period of vaccination the animals showed no signs of illness and X-ray photographs of their lungs taken from time to time revealed no evidences of pneumonia. 73 days after Monkeys 3 and 4 and 59 days after Monkey 10 had received their last intramuscular injection of virus, they were tested for immunity. Each of these animals and a normal control (Monkey 24) received intratracheally 2 cc. of a 1:100 dilution of a 10 per cent emulsion of livers and spleens from mice sick of psittacosis. The animals were observed carefully and X-ray pictures of all of them were taken on two occasions. The vaccinated monkeys did not become sick and had no fever; X-ray examinations revealed either no evidences or only slight evidences of pneumonia. The normal control monkey became sick, refused to eat, lost weight, became very weak, and had fever; X-ray pictures showed extensive pneumonia. The results of the experiment are graphically portrayed in Text-fig. 2 and are summarized in Table I.

The results of the experiment just described in detail and those of 5 other experiments summarized in Table I clearly indicate (1) that repeated intramuscular injections of active psittacosis virus in monkeys are relatively harmless and (2) that such injections produce in monkeys a heightened resistance to active psittacosis virus introduced intratracheally.

# Neutralizing Antibodies in the Sera of Vaccinated Monkeys

In addition to the demonstration of the fact that repeated intramuscular injections of active psittacosis virus lead to an increased resistance of monkeys to the active agent it seemed important that we determine whether such treatment is also followed by the appearance of neutralizing antibodies in the sera of vaccinated animals. To this end 8 monkeys were bled before the injections of virus were begun and again a month or more after their completion. The sera taken

before and after immunization were tested at the same time for their ability to neutralize psittacosis virus. Four sets of animals, 2 in each group, were handled in this manner, and the results obtained with one set will be given in detail.

Monkeys 13 and 14 were bled and the sera were pooled and placed in the ice box. Then the animals received 5 weekly intramuscular injections of active psittacosis virus in the manner described in the preceding section of the paper. 33 days after

TABLE III

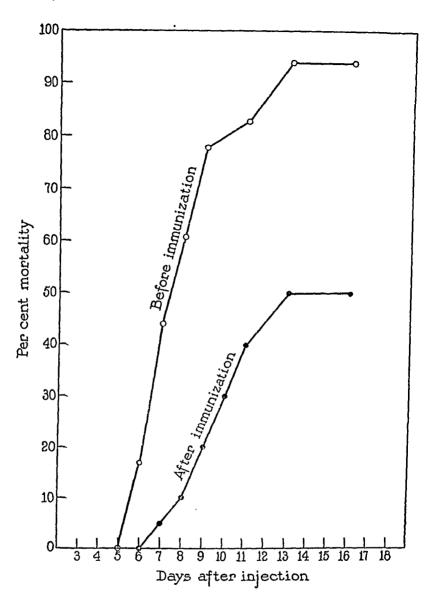
Summary of Neutralization Test Which Shows That Vaccination of Monkeys against
Psittacosis Leads to the Appearance in Their Sera of Neutralizing Antibodies

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of deaths	Average time of death	Neutraliza- tion index
	Se	rum before	e immunizat	ion + virus dilutio	ns	
10 <sup>-4</sup> 10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup>	4 4 5 5 5	4 4 5 4 17	94	6, 6, 6, 7 7, 7, 7, 7 8, 9, 9, 9, 13 8, 8, 11, 13	8.3	11.3
	S	erum after	immunizati	ion + virus dilution	as	
10-4 10-5 10-6 10-7	5 5 5 5	2 5 2 1		7, 8 9, 10, 10, 11, 11 9, 13 13		
	20	10	50		10.1	4.9

Neutralization index = percentage of deaths divided by average time of death.

the last injection the animals were bled again and the sera were pooled and inactivated at 56°C. for a half an hour. A neutralization test was made with the sera taken before and after immunization. The results of the experiment are summarized in Table III and Text-fig. 3.

The results of the above experiment and of 3 others summarized in Table I clearly indicate that repeated intramuscular injections of psittacosis virus led to the appearance of neutralizing antibodies in the sera of the vaccinated monkeys.



Text-Fig. 3. Graphic portrayal of the results of a neutralization experiment showing that vaccination of monkeys against psittacosis leads to the appearance of neutralizing antibodies against the virus in their sera.

## Immunity in Man to Psittacosis

Before proceeding with the vaccination of man against psittacosis it seemed advisable to look for evidence in regard to whether an attack of the disease protects a human being against reinfection or leads to the appearance of antibodies in the serum of a recovered individual. In the published reports no instances were found in which psittacosis attacked the same individual more than once. This fact, however, is not significant, because the attack rate of the disease is so low that one might not expect a second attack to occur in the same person even though an immune state did not result from the first one. In regard to the presence of antibodies in the sera of human beings convalescent from psittacosis Bedson (6) has shown that weak complement-fixing antibodies may be found. In previous work of ourselves (7) and of Bedson (6) few if any neutralizing antibodies were demonstrated in human convalescent serum. With our improved neutralization test, however, we decided to see whether we could obtain different results.

Neutralization tests were made on sera from 26 individuals. Many of the sera were tested several times and frequently two or more specimens of serum were obtained from an individual. Of the 26 people. 14 gave no history of having had psittacosis, while 12 either had had or were suspected of having had the disease 1 month to  $3\frac{1}{2}$  years previously. Of the 14 individuals with a negative history, 2 possessed and 12 did not possess demonstrable neutralizing antibodies in their sera. Of the sera from the 12 people with a positive or questionable history of psittacosis, 7 had no demonstrable neutralizing antibodies, 1 gave questionable results, and 4 were able to neutralize appreciable amounts of virus. The results of this work which is shown in the summary of 16 experiments in Table IV reveal the fact that the neutralization test is not a very reliable one for the diagnosis of psittacosis in retrospect. However, inasmuch as many of the individuals had recovered from the disease several years before the tests were made. and since it is a well recognized fact (8) that seasoned bird handlers are less likely to contract psittacosis than are novices in the trade, we were not discouraged but proceeded with the vaccination of human beings.

# Vaccination of Human Beings against Psittacosis

In approaching the problem of vaccination of human beings against psittacosis we decided that only volunteers would be vaccinated and that the first volunteer must be a physician who had not had the disease and who had no neutralizing antibodies for the virus in his serum.

Summary of Neutralization Tests in Which Mixtures of Human Sera and Psittacosis Virus Were Used TABLE IV

			Ć		i accon	212		
Test No.	Individual No.	History of psittacosis	Date of collection of serum	Date of test	Percentage of mice that died	Average day of death	Neutral- ization index	Interpre- tation of results
	3 2 1	- +, 1 ½ yrs. before -	Mar. 24, 1933 May 31, 1933 Mar. 17, 1933	June 28, 1933	100 67 96	8.0 9.3 8.1	12.5 7.2 11.9	1+1
2	45076	1111+	Feb. 14, 1933 July 3, 1933 July 3, 1933 July 3, 1933 July 3, 1933	July 12, 1933	88 89 89 89 89	8.9 7.5 7.9 9.3	10.1 11.3 12.2 11.2 7.3	1111+
8	7 8 0	+, 2 mos. before	Mar. 24, 1933 July 18, 1933 June 23, 1933	July 19, 1933	100 79 79	6.9 8.3 8.9	14.5 9.5 8.9	1++
4	2 9 10 10 5	Suspected case Suspected case " " " " "	June 23, 1933 July 26, 1933 Aug. 31, 1933 July 21, 1933 July 28, 1933 Sept. 4, 1933 July 3, 1933	Sept. 11, 1933	90 95 95 96	8.0 8.0 8.0 8.0 8.0	8.1 12.3 10.3 11.9 11.9	+11111
w	2 11 9 12 7	+, 3 ½ yrs. before Suspected case	Sept. 30, 1933 Sept. 24, 1933 Aug. 31, 1933 Sept. 16, 1933 Sept. 30, 1933	Oct. 3, 1933	42 75 84 88 70	10.2 9.3 9.7 9.1	8.1 8.7 9.7 7.9	+1111

			0.4 4 1033		8	8.6	8,7	i
	<u></u>	+, 3 \2 yrs. before	Oct. 1, 1933		20	0 0	7.1	ſ
	=	+	Sept. 24, 1933	Oct. 20, 1933	2 ;		. "	+
•	: ::	+. 3 14 vrs. before			જ લ	1.5	) +	
	; `	+	Sept. 30, 1933		22	13,0	C:	-
	•				91	8.8	10.3	ţ
	15	1			6	8.5	10.7	ſ
	6	Suspected case		0.4 30 1033	. 22	8.8	8.9	#
-	= :	+ -	Oct. 21, 1933	, , , , , , , , , , , , , , , , , , ,	16	8.9	10.2	í
	9 6	+, 3 >2 yrs. perore	Oct. 20, 1933		÷.	8.6	5.2	+
	•   :		Oct 31 1033		80	7.8	10.2	1
	7	+, 3 ½ yrs, before	Oct 31, 1933		8	8,6	9.3	t
	<u> </u>	+, 3 /2 yrs. Detore	Cont 27 1033	Nov. 13 1933	7.5	7.7	8.6	ı
œ	2	1	Sept. 41, 1933	2001 (01 1404)		0	7.7	+
_	2	+, 3 1/2 yrs. before	Nov. 1, 1933		3 :	7.0		1 -
	~	+	Oct. 20, 1933		8	0.0	7.0	+
	,		Oct 20, 1933		85	9.2	9.2	i
	- 1		Nov. 6, 1933		8	9.8	8,2	ı
	- 1	Before, during, and after vac-	Nov. 13, 1933		98 	0.6	8.9	ł
	٠ ٢	cination against psittacosis	Dec. 18, 1933		35	10,4	5,3	+
0			Jan. 3, 1934	Jan. 5, 1934	20	10.7	4.7	+
۸	:=		Sent. 4, 1933		75	9.7	7.7	ł
	: =	Before, during, and after vac-	σ:		22	6.6	7.1	ì
	: =	cination against psittacosis	, č		55	9.01	4.2	+
	20	+, 1 mo. before	Dec. 23, 1933		ž,	9.3	8.4	+

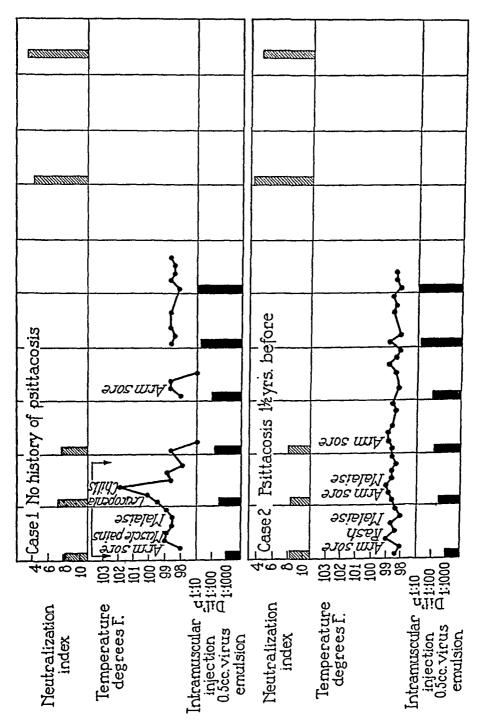
Neutralization index - percentage of deaths divided by average time of death. + indicates that the serum neutralized the virus.

- indicates that neutralization of the virus did not occur.

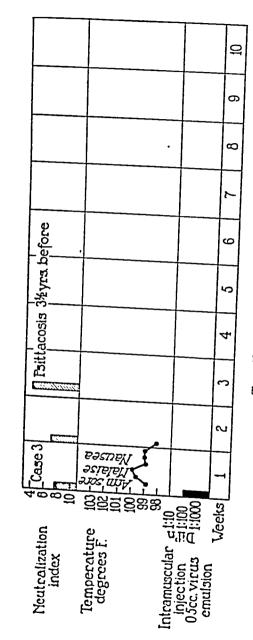
TABLE IV—Concluded

				monar				
Test No.	Individual No.	History of psittacosis	Date of collection of serum	Date of test	Percentage of mice that died	Average day of death	Neutral- ization index	Interpre- tation of results
10	21 22 22 22 23	After vaccination  Before, during, and after vaccination against psittacosis	Jan. 15, 1934 Jan. 15, 1934 Oct. 20, 1933 Nov. 6, 1933 Nov. 13, 1933 Dec. 18, 1933 Jan. 3, 1934	Jan. 15, 1934	75 90 84 80 71 45	11.3 8.5 10.2 9.6 8.9 13.5	6.6 10.6 8.2 8.3 8.0 3.3 4.7	+
11	21 21 6 6 6	After vaccination  Before, during, and after vaccination against psittacosis	Jan. 15, 1934 Jan. 15, 1934 Nov. 20, 1933 Dec. 4, 1933 Jan. 2, 1934 Jan. 18, 1934	Jan. 22, 1934	50 75 75 75 65 65	12.1 7.8 8.4 8.0 8.5 9.9	4.1 9.6 10.7 9.4 7.6 6.6	+111++
12	22 8 8 8 8 8	After vaccination  Before, during, and after vaccination against psittacosis	Jan. 18, 1934 Feb. 1, 1934 Nov. 20, 1933 Dec. 4, 1933 Jan. 2, 1934 Jan. 18, 1934	Feb. 2, 1934	42 40 65 50 40	10.8 9.4 9.2 9.2 9.4 10.9	3.9 4.3 7.1 7.1 3.3	++11#+
13	23 24 24 27 24 24 24 24 24 24 24 24 24 24 24 24 24	After vaccination  " " " " " " " " "	Feb. 8, 1934 Feb. 7, 1934 Feb. 7, 1934 Feb. 7, 1934 Feb. 7, 1934 Feb. 7, 1934	Feb. 9, 1934	70 70 60 70 75 85 80	10.1 8.3 10.1 10.4 10.6 13.0	6.9 8.4 5.9 6.7 5.2 4.6	+ 1 + + + +

111++++	1+	1 <del>1</del>	- 1	
9.6 8.7 8.9 7.1 6.4 5.8	6.9 4.6	8.4	7.7	
8.9 10.3 10.1 9.1 8.6 10.3 10.9	9.2	8.9	9.2	
85 90 90 53 55 60 60	63	75	45	
Feb. 24, 1934	Mar. 8, 1934		Apr. 10, 1934	
Jan. 4, 1934 Jan. 4, 1934 Jan. 3, 1934 Jan. 18, 1934 Feb. 8, 1934 Jan. 3, 1934	Jan. 4, 1934	Mar. 6, 1934	Jun. 29, 1934 Feb. 21, 1934 Mar. 6, 1934	
After vaccination After vaccination	nofore vaccination	After vaccination	During vaccination After vaccination	After vaccination
25 26 23 23 5	7 2	22	26 26	56
=		15	9	



TEXT-FIG. 4-Continued on Next Page



Text-Fig. 4. Summary of results obtained by means of vaccination of 3 human beings against psittacosis. Text-Fig. 4-Concluded

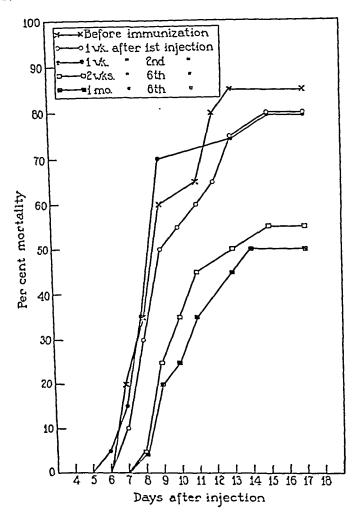
It was further decided that, inasmuch as the period of vaccination is 5 or 6 weeks, during which time the individual might have a cold, the procedure must be safe even under such circumstances. A suitable volunteer presented himself and we waited until he developed a common cold accompanied by a bronchitis before proceeding with the vaccination. As a control another volunteer, a physician, who had had psittacosis  $1\frac{1}{2}$  years previously and who had neutralizing antibodies for the virus in his serum was also subjected to vaccination.

Serum was collected from the volunteers before the inoculations of virus were begun. Then each individual received 6 weekly intramuscular inoculations of active psittacosis virus. The amounts of virus and the order of their administration were 0.5 cc. of the following dilutions of 10 per cent emulsions of livers and spleens of infected mice: 1:1000, 1:500, 1:200, 1:100, 1:20, 1:10. The first and last inoculums contained sufficient virus to kill 10,000 and 10,000,000 mice respectively. 1 and 2 weeks after the first injection and 2 and 4 weeks after the last inoculation specimens of serum were collected from each individual for neutralization tests.

The intramuscular injections of psittacosis virus into the two volunteers resulted in no considerable ill effects. The details of the experiment are shown under Cases 1 and 2 in Text-fig. 4. The person (Case 1) who had not had psittacosis previously had two chills accompanied by a fever of short duration after the second inoculation. The neutralization indices of the sera taken before, during, and after vaccination are recorded in Table IV (Case 1 of Text-fig. 4 is Individual 7 of Table IV, and Case 2 is Individual 2) and plotted in Text-fig. 4. The results of the neutralization tests on specimens of serum collected from Case 1 are more fully detailed in Table V and are graphically portrayed in Text-fig. 5. It is evident from the results of these tests that neutralizing antibodies either appeared or increased in amount as the result of vaccination.

While the work was in progress we had the opportunity to determine what effect one injection of a moderate amount of active virus would have on an individual who had had psittacosis  $3\frac{1}{2}$  years previously and who had no neutralizing antibodies in his serum. Blood was collected and then 0.5 cc. of a 1:100 dilution of a 10 per cent emulsion of livers and spleens from infected mice was given intramuscularly. Specimens of blood were again collected 1 and 2 weeks after the inocu-

lation. In Text-fig. 4 (Case 3), Table IV (Individual 11), and Text-fig. 6 the results are shown. The inoculation led to no more than a



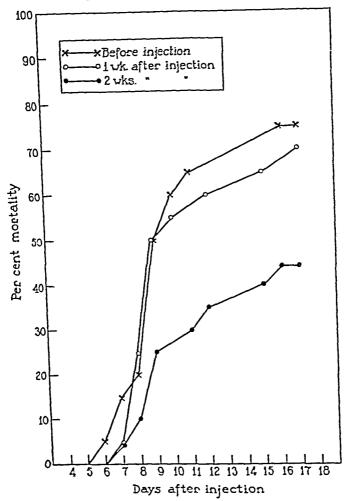
Text-Fig. 5. Graphic portrayal of the results obtained in a neutralization test on specimens of serum collected from Case 1 before, during, and after vaccination against psittacosis.

slight discomfort in the individual but induced neutralizing antibodies to appear rapidly in his serum. In this instance the response had the

TABLE V
Summary of a Neutralization Test Which Shows That Vaccination of a Human Being
(Case 1) against Psittacosis Led to the Appearance of Neutralizing Antibodies
in His Scrum

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of deaths	Average time of death	Neutraliza- tion index			
	Se	rum before	e immunizat	ion + virus diluti	ons	<del>-</del>			
10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5 5 5 5	5 5 4 3		7, 7, 7, 7, 9 8, 8, 8, 9, 9 9, 9, 12, 12 11, 12, 13					
	20	17	85		9.2	9.2			
	Serı	ım 1 wk. a	fter 1st inje	ction + virus dilu	tions				
10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5 5 5 5	5 5 4 2		7, 8, 8, 9, 12 7, 8, 9, 9, 9 8, 10, 11, 13 13, 15					
	20	16	80		9.8	8.2			
	Seru	m 1 wk. af	ter 2nd inje	ction + virus dilu	tions				
10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5 5 5 5	5 5 4 2		6, 7, 7, 8, 9 8, 8, 9, 9, 9 8, 9, 9, 13 9, 15					
	20	16	80		9.0	8.9			
	Seru	m 2 wks. a	fter 6th inje	ction + virus dilu	tions				
10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5 5 5 5	5 4 2 0		8, 9, 9, 9, 13 9, 10, 11, 11 10, 15					
	20	11	55		10.4	5.3			
	Seru	m 4 wks. a	fter 6th inje	ction + virus dilut	tions				
10 <sup>-5</sup> 10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5 5 5 5	5 3 2 0		8, 9, 10, 11, 13 9, 9, 11 13, 14					
	20	10	50		10.7	4.7			

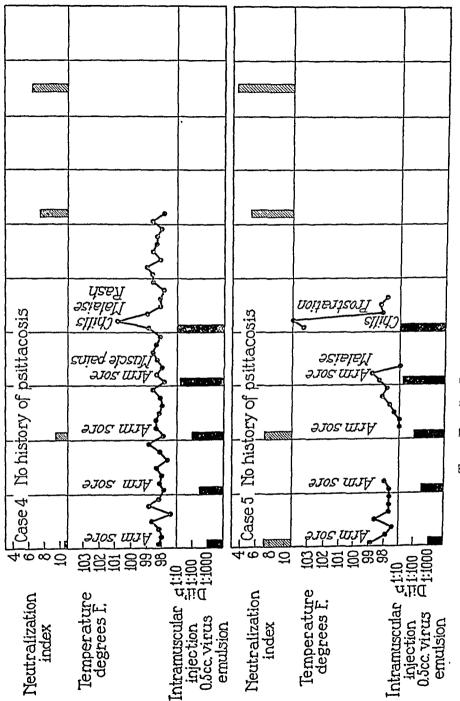
characteristics of a secondary rather than a primary reaction to an antigen. This finding leads us to believe that individuals who have



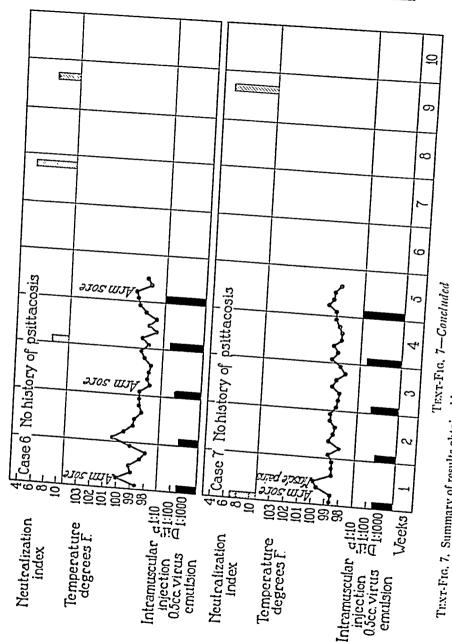
TEXT-FIG. 6. Graphic portrayal of the results obtained in a neutralization test on specimens of serum collected from Case 3 before and after one intramuscular injection of psittacosis virus.

had psittacosis possess an appreciable degree of immunity even though they have no demonstrable antibodies in their sera.

Four other volunteers, three laboratory boys and one physician (Cases 4, 5, 6, and 7 of Text-fig. 7 which are respectively Individuals

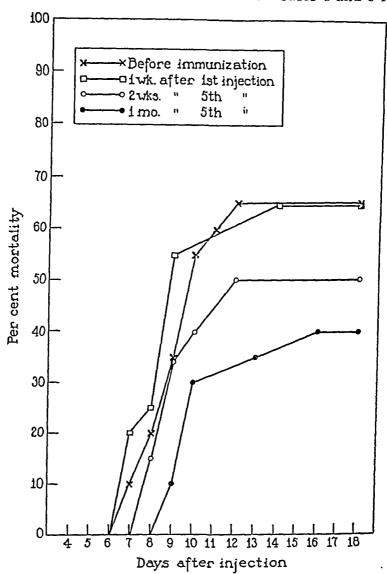


TEXT-Fig. 7-Continued on Next Page



Text-Fig. 7. Summary of results obtained by means of vaccination of 4 human beings against psittacosis,

6, 5, 26, and 25 of Table IV), have been vaccinated in a manner similar to that described for Cases 1 and 2. Cases 4 and 5 received



Text-Fig. 8. Graphic portrayal of the results obtained in a neutralization test on specimens of serum collected from Case 4 before, during, and after vaccination against psittacosis.

5 weekly injections of 0.5 cc. of the following dilutions of 10 per cent emulsions of infected livers and spleens: 1:1000, 1:500, 1:100, 1:20, 1:10. For Cases 6 and 7 the following dilutions were used: 1:500,

1:500, 1:100, 1:50, 1:10. The individuals suffered no marked ill effects from the injections. In two instances (Cases 4 and 5), however, chills and fever similar to those that occasionally occur after typhoid vaccination were experienced following the last inoculation. The results of the neutralization tests shown in Table IV and Text-figs. 7 and 8 clearly indicate that neutralizing antibodies also appeared in the sera of these volunteers as the result of vaccination.

### DISCUSSION

The results of our work indicate that when psittacosis virus produces a pneumonia in man its portal of entry is the upper respiratory tract. The fact is plain that man is very unlikely to contract psittacosis pneumonia as the result of the subcutaneous or intramuscular introduction of active virus. It is not known whether the individuals who have received repeated intramuscular injections of active virus possess an increased resistance to psittacosis in addition to the neutralizing antibodies in their sera, and, if they do possess such a heightened resistance, there is no evidence in regard to the length of time they will retain it. Nevertheless, in view of the results obtained with monkeys. one is justified in assuming that a certain degree of protection against psittacosis was afforded the human volunteers by means of vaccination. Protection of an individual by means of the introduction of an active infectious agent through a portal of entry other than the one used in nature is an old and established procedure. For instance, variolation was employed for many years as a protective measure against smallpox.

### SUMMARY

Monkeys that have recovered from psittacosis pneumonia have an increased resistance to infection with the virus and possess neutralizing antibodies in their sera.

Large amounts of active psittacosis virus can be introduced intravenously and intramuscularly into monkeys without the production of a serious infection such as pneumonia. Relatively small amounts of virus introduced intratracheally into monkeys usually lead to psittacosis pneumonia.

Monkeys vaccinated intramuscularly with unattenuated psittacosis

virus have an increased resistance to the active agent and possess neutralizing antibodies in their sera.

The intramuscular introduction of active psittacosis virus in moderate amounts into human beings is relatively harmless, and repeated inoculations lead to the appearance of neutralizing antibodies in the sera of vaccinated individuals.

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# BIOCHEMICAL STUDIES ON THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

## I. ISOLATION AND CHARACTERIZATION OF FIBRINOLYSIN

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The fibrinolytic activity of hemolytic streptococci designates the capacity of cultures of the organisms to liquefy rapidly human fibrin clot (1). The lytic action is referable to the presence in the culture of an extracellular substance, characteristically elaborated by those strains of hemolytic streptococci which are derived from patients suffering with acute streptococcus infections.

Investigation of the phenomenon has included studies concerning the mechanism involved in the reaction and consideration of a possible relationship to the more general problem of infection and resistance (2).

The biochemical studies to be described in this and the succeeding communication deal with two aspects of the phenomenon. This report is concerned with the isolation and characterization of the active fibrinolytic principle elaborated by the organisms. Methods are described by which the lytic substance may be removed from culture filtrates, concentrated, and partially purified. Some of the physical properties of the active preparations are described.

The second paper (3) deals with the biochemical action of the lytic substance and the nature of the end-products.

The phenomenon of fibrinolysis induced by the bacterial agent consists of a rapid change of fibrin from its solid clotted form to a soluble substance. The transformation, therefore, as it occurs in vitro, is readily visible, since the coagulated gel is not disturbed by inversion of the test tubes and the process of liquefaction converts the

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clot to a limpid solution. The end-point of a fibrinolytic test is established by determining the occurrence of complete dissolution.

Throughout the study, the length of time necessary to effect lique-faction is employed as a rough quantitative measure of the amount of fibrinolytic substance present in a test preparation of bacterial material. For example, when one sample liquefies a given amount of fibrin in 2 minutes and another sample requires 1 hour to dissolve the same amount of fibrin, this difference in time is used as a relative measure of fibrinolysin in the two preparations. Furthermore, when 0.001 cc. of one sample causes lysis just as quickly as does 0.5 cc. of another sample, the former preparation is deemed to contain more fibrinolytic substance than the latter. The complications involved in measuring quantity on the basis of time are appreciated but the method is, nevertheless, applicable to the purpose of the experiments which follow.

### Materials and Methods

In the earlier report (1) it was demonstrated that when fibrinogen, chemically isolated from human plasma, was clotted with preparations of thrombin, also derived from human blood, lysis occurred with extraordinary rapidity. For the purposes of the experiments with which this study is concerned, it has been advantageous to use fibrinogen and thrombin solutions, thus eliminating some of the fractions of whole blood, which do not participate in the reaction.

It has also been demonstrated (1) that, even though rabbit plasma clot is resistant to dissolution, rabbit fibrinogen, when coagulated with human thrombin, forms a fibrin substrate which is highly susceptible to lysis. Rabbit fibrinogen-human thrombin combinations have been found to be uniformly satisfactory in tests concerning the activity of the fibrinolytic substance and have been regularly employed for this purpose.

Fibringen and Thrombin Solution.—The fibringen and thrombin solutions were prepared according to the methods described in the earlier report (1).

Cultures.—A strain of beta hemolytic streptococcus, designated Co, has been employed in these studies. The organism was grown in beef infusion broth which contained 2 per cent NaCl, 1 per cent peptone, and 0.05 per cent dextrose. The broth was adjusted to pH 7.6 but did not contain additional buffers. Incubation of the culture was carried out for 14 to 18 hours. At the end of the incubation period the culture was centrifuged and the supernatant fluid was filtered through a Berkefeld Type V candle. The sterile filtrate was then tested for fibrinolytic activity in the manner described below. Only highly potent material was used.

Description of Test.—Titration consisted in testing the potency of progressive dilutions of material containing streptococcal fibrinolysin. The test for the fibrinolytic potency of a preparation, unless otherwise stated, was carried out as

follows: To a series of appropriate dilutions of the test solution sufficient physiological saline solution was added to bring the volume of each to 0.7 cc. 0.2 cc. of a solution of rabbit fibrinogen was added and thoroughly mixed. Then 0.1 cc. of a human thrombin preparation was added as rapidly as possible after the introduction of fibrinogen, and each tube was well shaken. The test was then placed in the water bath at 37.5°C. and the time of coagulation and liquefaction of each clot was recorded. When fresh preparations were employed the coagulation time, determined in the usual manner by inverting the tube, rarely exceeded one minute. All solutions were warmed to room temperature prior to using them in a test.

Other technical procedures are given in the body of the report according to the type of experiment which was performed.

## Isolation, Concentration, and Partial Purification of the Fibrinolysin of Hemolytic Streptococci

The fact that the streptococcal fibrinolysin is copiously present in the cell-free Berkefeld filtrates of broth cultures of hemolytic streptococci has greatly facilitated studies of the active principle. In the experiments to be described, therefore, highly active, sterile filtrates were always utilized, thus excluding the possible complications associated with the growth and metabolism of living cells.

Four methods have been used for the purpose of isolating and concentrating the active fibrinolytic principle. They are: (a) concentration under reduced pressure; (b) precipitation with organic solvents; (c) concentration by vacuum dialysis; (d) specific adsorption and elution.

(a) Concentration under Reduced Pressure,—400 cc. of active culture filtrate were concentrated in racuo. During the distillation the water bath was maintained at 40°C., and the vacuum was obtained with an oil pump. The receiver was cooled in an ice-salt bath. Under these conditions the volume of filtrate was reduced to 40 cc. in 4 hours at which time the operation was terminated. The tenfold concentrate, which was deeply colored, was then tested for fibrinolytic activity and its potency compared with that of the original solution. The results are as follows:

Dilution of solution, cc	0.5	0.1	0.05	10.0	0.065	10000	0.0005
Original filtrate	2	min. 5 2	7:in. 10 2	7:in. 31 9	#:in. 45 27	mis. — 40	rifs. — 63
Concentrate (after 24 hrs.)	5	7	45	-	-	-	-

<sup>\*</sup> In the tabulations, figures indicate not only that dissolution occurred but also the number of minutes required for lysis; the symbol (-) indicates that no lysis occurred in 2 hours.

From these results it can be seen that not only has the concentrate retained its activity after the treatment but that a definite increase, at least fivefold, has been obtained. The potency of the preparation did not remain constant and an appreciable decrease in titer was observed after storage for even so short a time as 24 hours in the ice box. Although the results of these preliminary experiments indicate that concentration may be effected under reduced pressure, the instability of the product prepared in this manner has rendered its use impractical. Consequently, the method has not been regularly employed.

(b) Precipitation with Organic Solvents.—The fibrinolytic principle, together with other organic materials and inorganic salts, was precipitated when the culture filtrate was treated with several volumes of either acetone, methyl alcohol, or ethyl alcohol. The latter precipitant has been regularly employed. The procedure was as follows:

Active culture filtrate, 750 cc., was cooled to 0°C. in an ice-salt bath. Three volumes of cold 95 per cent alcohol (2,250 cc.) were slowly added with constant stirring. The mixture was permitted to stand at 0°C. for an hour. The material readily flocculated and was collected by centrifugation. The grey colored precipitate was stirred with ice-cold absolute alcohol, transferred to a small Buchner funnel, and washed alternately with cold absolute alcohol and cold anhydrous ether. After a final washing with ether the amorphous material was dried in a vacuum desiccator which contained phosphoric anhydride and chips of paraffin. When thoroughly dry, the material was ground to a fine powder and was stored over CaCl<sub>2</sub>. The yield was 1.88 gm. of solid substance from 750 cc. of culture filtrate, or approximately 2.5 mg. per cc.

The dry preparation of the fibrinolytic substance dissolved slowly but completely in physiological saline and in dilute acid pH 6.0. Solution in M/20 phosphate buffer, pH 8.0, left a small amount of insoluble residue which was, however, shown to contain none of the fibrinolytic principle. A solution prepared by dissolving 2.5 mg. of the dry powder in M/20 phosphate-buffered physiological saline, pH 7.2, was tested for fibrinolytic activity and compared to the original filtrate. In addition, a more concentrated solution containing 10 mg. per cc. was prepared and tested. The results were as recorded:

Dilution of solution, cc	0.5	0.05	0.005	0.0025	0.001
Original filtrate	3	min. 5 9 5	min. 21 34 20	min. — — 41	min

From these results it is seen that practically all of the fibrinolysin is precipitated by treatment of active culture filtrates with alcohol. The activity of the dry preparations is readily demonstrated by testing appropriate solutions of the precipitate. Thus, a solution which contained the dry material in a concentration approximately equivalent to that of the original culture filtrate (calculated upon the basis of the yield) compared favorably with the latter in respect to fibrinolytic potency. A more concentrated solution of the dry material was proportionally more potent in promoting the dissolution of the fibrin clots. Since the solubility of such a dry preparation is relatively low, the precipitation method has not proved to be of particular value from the point of view of the concentration of the fibrinolysin. The method is useful, however, as a means of obtaining a quantity of active material for preservation in the dry state. The stability of the preparations is shown by the fact that material stored for 11 months in a desiccator at room temperature lost none of its original potency.

Although consistently highly active, the more concentrated solutions of the dry preparations have occasionally inhibited the coagulation of plasma by CaCl<sub>2</sub>. This inhibition may be attributable to the presence, in the fibrinolytic preparation, of peptone, which has been variously reported to possess anticoagulant properties. Many preparations have been obtained, however, not having an anticoagulant effect.

(c) Concentration by Vacuum Dialysis.—Active culture filtrate, 750 cc., was placed in a vacuum dialyzer similar to the apparatus described by Simms (4). Dialysis against distilled water under a negative pressure of 40 cm. of mercury was permitted to proceed for 20 hours. During this time the volume of the filtrate was reduced to 25 cc. The solution was tested for fibrinolytic activity, and compared with the original filtrate. The results were as follows:

Dilution of solution, cc	0.5	0 05	0.005	0.0005	0 0001
	ris.	mis.	min.	rin.	min.
Original filtrate	7	8	90	-	
Concentrated		2	4	27	76
Concentrated (7 days old)	2	2	4	38	95

As can be readily seen, concentration was even more successful by this method than by the use of reduced pressure (vacuum distillation). Furthermore, the solution, stored in the ice box for a week, did not decrease appreciably in titer. The stability of the solution over a longer period was not tested.

(d) Specific Adsorption and Elution.—A variety of inert materials were tested for the property of specific adsorption of the fibrinolytic principle from culture filtrates. Among these kaolin, talcum, charcoal, and the diatomaceous earths were without effect. It was possible, however, to obtain specific adsorption of the active principle upon alumina. The polyaluminum hydroxide B of Willstätter (5) has proved of particular value for this purpose.

Active culture filtrate, 2,500 cc., was intimately mixed with the aluminum hydroxide gel prepared from 250 gm. of aluminum sulfate (Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·18 H<sub>2</sub>O). mixture was incubated at 37.5°C. for an hour, during which time it was frequently stirred. The alumina was removed by centrifugation. That adsorption had taken place was indicated by the fact that the supernatant fluid, when properly tested, possessed no fibrinolytic activity. The alumina was then suspended in 1,000 cc. of physiological salt solution, thoroughly stirred, and centrifuged. The supernatant saline solution contained a considerable amount of pigment but was shown by test to have extracted none of the fibrinolytic principle. The washing of the gel was repeated twice with physiological salt solution and twice with distilled water. The alumina was then thoroughly triturated with 500 cc. of a M/10 phosphate buffer solution which was prepared by dissolving 35.8 gm. of Na<sub>2</sub>HPO<sub>4</sub>·12 H<sub>2</sub>O in 950 cc. of distilled water, adjusting the pH to 7.3 by the careful addition of a little concentrated HCl, and diluting to a volume of 1,000 cc. The alumina suspension was incubated for 1/2 hour at 37.5°C. and was centrifuged. The supernatant fluid was highly active in promoting the dissolution of the fibrin. The second and third elutions were carried out in the same manner. The fourth elution fluid contained only a trace of the fibrinolytic active substance and was discarded. second, and third elution fluids were combined and filtered through a Berkefeld filter. The clear, highly active eluate contained in 1,500 cc. was then concentrated in the Simms dialyzer to a volume of 50 cc. Since the dialysis required approximately 16 hours, sterility was insured by the addition of a little toluene before beginning the procedure. The resulting clear, slightly brown concentrate was tested for fibrinolytic potency and compared to the original culture filtrate.

A 5 cc. portion of the concentrate was evaporated to dryness in vacuo over phosphoric anhydride. The product, dark brown scales, dissolved readily in 5 cc. of physiological salt solution and was tested for the fibrinolytic activity in the usual manner. A summary of the tests carried out upon the various solutions which were obtained during the experiment is presented below:

Preparation No.	Material, cc.	0.5	0.05	0.003	0.001	0.0005	0.0001
		min.	min.	rin.	nin.	min.	min.
ı	Original filtrate	7	8	36	-		
II	Preparation I, adsorbed	-	_	—	-		
$\mathbf{III}$	Saline washing			—	-	-	—
IV	Elution 1	2	4	15	50		-
v	Elution 2	2	2	8	34	63	
VI	Elution 3	6	9	41		—	
VII	Elution 4	15		<b> </b>	-	1 —	
VIII	Combined Elutions 1, 2, 3	2	2	8	37	54	
IX	Preparation VIII; concentrated	2	2	5	16	23	64
X	Preparation IX; stored 14 days	2	2	7	20	35	50
XI	Preparation IX; dried, redissolved	2	2	6	19	25	55

The adsorption of the fibrinolysin upon the alumina and the application of appropriate elution and concentration processes resulted in the most potent preparations of the fibrinolytic principle which have thus far been obtained. Although the method of comparing the solutions by titer cannot be considered accurately quantitative, inspection of the results indicates that the various steps in the procedure may be performed with but little loss of the active material originally present in the filtrate.

Throughout the experimental work which has been described it was frequently observed that different culture filtrates varied in their content of fibrinolysin. The difference was not apparent when 0.5 cc. of the filtrate was tested but became increasingly manifest when higher dilutions of the active filtrate were used. The fact becomes important when the potencies of the various preparations of the fibrinolysin are to be compared. An experiment was therefore carried out in which the three most favorable methods of concentration were applied to portions of the same culture filtrate. The results of the experiment are presented in Table I.

The above experiment, which was carried out with a single culture filtrate, leaves no doubt that the active fibrinolytic principle of hemolytic streptococci may be successfully concentrated.

# Properties of the Fibrinolytic Principle

Effect of Heat on Fibrinolysin.—The resistance of the streptococcal fibrinolysin to thermal inactivation has been found to be conditioned

by several factors relating to the test material. The experiments have, therefore, been carried out not only with culture filtrates, but also with concentrated preparations isolated by the methods just described. A summary of the results of a series of heating experiments is presented in Table II.

TABLE I

Comparison of Different Preparations of the Fibrinolytic Principle from Culture

Filtrate 110

					Amo	unt of	prepa	ration			
No.	Preparation	0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.001 cc.	0.0005 cc.	0.00025 cc.	0.0001 cc.
		min.	min.	min.	min.	min.	min.	min.	min.	min.	min.
I	Original culture filtrate	*7	8	8	22	36				l —	
II	I, alcohol precipitate, 2.5	3	5	8	21	32			-		-
:	mg. per cc.										
III	I, alcohol precipitate, 10 mg. per cc.	2	2	2	8	16	22	38		-	_
IV	I, concentrated and dialyzed	2	2	2	4	4	7	18	27	41	76
v	First elution from ad- sorbed I	2	2	4	9	15	38	50		-	
VI	Second elution from ad- sorbed I	2	2	2	5	8	25	34	63	_	
VII	Third elution from ad- sorbed I	6	9	9	18	41	-	-	-	-	
VIII	V, VI, and VII, combined,	2	2	2	4	5	9	16	23	34	64
IX	VIII, dried and redissolved	2	2	2	4	6	8	19	25	40	55

All tubes incubated in water bath at 37.5°C.

From the data presented in Table II it is apparent that the fibrinolysin contained in the broth filtrates and in the adsorbed, eluted, and concentrated preparations, is particularly resistant to thermal inactivation. In interesting contrast to this is the fact that the activity of solutions of the alcohol-precipitated material is heat-labile, being partially inactivated after exposure to a temperature of 57°C. for ½ hour, and completely destroyed after 1 hour at this temperature.

<sup>\*</sup> In Tables I to IV, figures represent time in minutes of interval between clot formation and complete liquefaction; the symbol (—) indicates that no liquefaction occurred in 2 hours.

TABLE II

	Coort	100 C:	0.5 0.1 0.05 0.01 0.005 0.0 0.0 0.5 0.1 0.05 0.0 0.0 0.005 0.5 0.0 0.0 0.0 0.0	x x x x 10 9 15 47 — x x x x 4 3 10 15 x x x x 4 3 3 12 16 x x x x 4 3 8 16 20 x x x x 4 9 20 25 x x x 4 9 20 25
	$\ $		0.05 0.01 0.005 cc. cc. cc. min. min. min.	5 x 2 x 2 x
=			0.01 cc.	0° × ~ × ∞ ×
vatio		75°C.	0.05 Rin.	0 × + × × ×
nacti	$\ $		1.6 1.1 1.1 1.1 1.1	∞ × × \ \ ×
I lou			0.5 cc.	× × - × × ×
11,047	311		0.05 0.01 0.005 cc. cc. min.	32 32 32 33 34 35 35 35 35 35 35 35 35 35 35 35 35 35
,	2 10		0.01 cc.	0.844444
<b>4</b>	111011	2.2	0.05 cc.	ο ο - κ
TABLE II	cpar		0.1 cc.	8 - 1 - 3 - 1 - 3
<u>;</u> :	ic Pr		0.5 cc.	EEKXXX4231
,	Fibrinolyl		Duration of heating	1 hr. 2 hrs. 1 hr. 2 hrs. 1 hr. 2 hrs. 15 min. 30 min. 45 min.
	Resistance of Fibrinolytic Preparations to Ancient		Preparation	Culture filtrate

All tubes in water bath at 37.5°C.
The symbol (x) indicates that the test was not set up.

The reason for the abrupt change in thermal stability following precipitation by alcohol is not clear. The fact that the more potent and, probably, much purer preparations, obtained by the adsorption process, are heat-stable, appears to more closely reflect the correct characterization of the fibrinolysin.

In studies on the effect of heat on the fibrinolysin, different samples of filtrates have not always given results identical with those presented in Table II, especially when higher temperatures were used. Consequently, the experiments were repeated with special consideration being given to the hydrogen ion concentration of the solutions.

Procedure.—Freshly prepared broth was inoculated with streptococci and incubated for 14 hours. The culture was filtered and four 15 cc. portions of the filtrate were adjusted to pH 5.5, 6.5, 7.5, and 8.5, respectively, with accurately measured volumes of N/10 HCl and N/10 NaOH. The tubes were placed in the water bath at 37.5°C. Similar series of preparations were incubated at temperatures of 57°, 75°, and 100°C. (boiling water bath). At intervals of 30 minutes, 1 hour, and 2 hours a test portion of each solution exactly equivalent to 2.5 cc. of the original culture filtrate was withdrawn, cooled rapidly to room temperature, and carefully neutralized with a predetermined volume of N/10 NaOH or N/10 HCl. The test portion was then accurately diluted to a volume of 5 cc. with M/20 phosphate buffer, pH 7.3. Each test solution thus represented a 1 in 2 dilution of the original filtrate. The various solutions were tested at once against the usual rabbit fibrinogen-human thrombin clots. The results of the experiment are presented in Table III.

From Table III it is evident the potency of the culture filtrate is appreciably decreased only upon exposure to temperatures of 75–100°C. At these temperatures the hydrogen ion concentration of the solution is an important factor in the thermal destruction of the fibrinolytic agent. Thus, at pH 5.5 and 8.5 it is seen that the fibrinolytic agent is almost completely destroyed by exposure to a temperature of  $100^{\circ}$ C. for a period of  $\frac{1}{2}$  hour. In solutions at a pH of 6.5 to 7.5, however, the potency of the active substance is decreased only upon longer exposure to this temperature.

Even though the results presented in Tables II and III indicate important factors (method of isolation, and pH) which determine heat stability of fibrinolysin, still other minor discrepancies may be encountered. These latter variants are not yet understood, but seem to be associated with age and composition of the culture medium.

TABLE III

Influence of Hydrogen Ion Concentration of Culture Filtrates on Thermal Inactivation

	0.01 cc.	min.		l	l	ļ	56	8	ĺ	. (	38	1	i	I	1	1	
100°C.	0.025	-		1	i	1	7	17	:	!	17	27	i	29	ſ	1	
100	0.05		•	1	1	1	9	13	:		13	23	ſ	70	1		
	0.25			21	ļ	1	L/S	7		7	ဖ	2	96	91	1		
	0.01		14111.	915	20	35	2 02	2,5	3:	<b>*</b>	6	10	#	9	75	?	i
75°C.	0.025	3	min.	20	19	1.7	=	: :	7 6	707	7	7	10	7		3 5	2
75	0.03	ان	min.	10	Ξ	=	; 0	9 0	o ;	÷	ις.	ی		, v.	α	o ;	=
	0.25	į	min.	9	1-		, L	,	o :	_				· ·	. ·		8
	0.01	ઇ	min.	Ξ	28	30	9 :	3 9	≘	=	0	, α	; c	, 0	٥٤	=	=
s7°C.	0.025	છું	min.	~	. 2	: :	3 4	٠,	: <u> </u>	~	٠		3 6	٠ ،	0 0	0	တ
57	0.05	Ġ.	min.	٠	, 0		- ·	ימ	S	v	v	۰ د	3 4	o u	<u>.</u>	0	7
	0.25	છું	min.	4	. 1/	· ·	٠.	S.	m		• •	٠,	ი -	÷,	ν, •	•	÷
; <b> </b>	10.0		min.	c		٠ <u>:</u>	2	22	=	7	- 0	0 0	~ 0	» с	χ 1	_	0
37°C.	0 0 25	<u>છ</u>	min.	,	، ،	`	<u>ه</u>	157	9	٠	:	c :	• •	۰ د	c ·	<u>۔</u>	
	200	} છં 	1,1		۰ ،	c 	v,	v,	v,	_	· ·	÷ ,	ر د	ر د در	٠,	÷	v.
-	3,0	ું કું —	1	•	÷ ·	÷	<del></del>	~	S	,	· ·	. ب	<u>.</u>	m ·	<del></del>	÷	~
	He				ر د	5.5	S	6.5	6.5		0 1	7.5	7 5	7.5		×.	8.5
	Puration of Leating				.30 min.	- Pr	2 hra.	30 min.	1 hr.	. ,	7 II.	30 min.	_ ]: ]:	2 hr.	30 min.	1 11.	2 hrv.

All tubes incubated in water bath at 37.5°C.

It may be seen, therefore, that the problem of thermal inactivation is not simple, but the experiments just described serve to characterize the lytic agent as heat-stable.

Effect of Proteolytic Enzymes upon the Fibrinolysin.—In view of the fact that the solutions of the fibrinolysin invariably gave a positive biuret protein test, it was considered of interest to determine the action of proteolytic enzymes upon the active fibrinolytic principle. For this purpose trypsin and papain have been employed.

TABLE IV

Action of Trypsin and Papain upon the Fibrinolysin

Preparation	Duration of	Amount of preparation			
reparation	incubation	0.5 cc.	0.1 cc.	0.05 cc.	
		min.	min.	min.	
Fibrinolysin + saline (control)	None	3	8	12	
Fibrinolysin + saline (control)		3	7	10	
Fibrinolysin + saline (control)		3	7	11	
Fibrinolysin + trypsin	None	4	8	14	
Fibrinolysin + trypsin	30 min.	17	-		
Fibrinolysin + trypsin	1 hr.				
Fibrinolysin + papain	None	4	9	12	
Fibrinolysin + papain	30 min.	10	25	87	
Fibrinolysin + papain	1 hr.	-			

All tubes incubated in water bath at 37.5°C.

Procedure.—Dry, alcohol-precipitated fibrinolytic material was dissolved in M/20 phosphate buffer of pH 7.0. To 4 cc. of this solution was added 1 cc. of a 0.05 per cent solution of commercial trypsin. A similar preparation contained 1 cc. of a 0.05 per cent solution of papain in place of the trypsin. The papain had been previously activated by a trace of HCN. A control solution was prepared by adding 1 cc. of physiological salt solution to 4 cc. of the fibrinolytic solution. The mixtures were incubated at 37.5°C. Portions of each mixture were withdrawn at intervals of 30 minutes and 1 hour and tested in the usual manner. The results of the test are shown in Table IV.

From Table IV it is seen that both trypsin and papain rapidly and completely inactivated the fibrinolysin. This result, considered in connection with the facts that the fibrinolysin is non-dialyzable and is readily adsorbed upon alumina, strongly suggests that the active fibrinolytic principle may be protein in nature. This possibility

corresponds to certain recent developments in enzyme chemistry which have shown that at least three enzymes, urease, pepsin, and trypsin, are proteins (6).

Demonstration of the Fibrinolytic Principle in Reaction Mixtures.— The following experiments were carried out to determine whether or not the fibrinolytic principle is adsorbed by the dissolved fibrin and is demonstrable in the presence of the reaction products.

Procedure. Experiment I.—To 0.2 cc. of rabbit fibrinogen, diluted with 0.2 cc. of physiological saline, 0.5 cc. of a fibrinolytic preparation was added, the activity of which had been shown by titration to be such that 0.0001 cc. caused dissolution, in a usual test in 50 minutes. This mixture was placed in a water bath at 37.5°C. and coagulation was induced by the immediate addition of 0.1 cc. of human thrombin. The time at which coagulation was complete and the interval in minutes between coagulation and liquefaction of the clot were recorded. Immediately after liquefaction of the clot 0.5 cc. of the mixture was withdrawn and added to a tube which contained 0.2 cc. of human fibrinogen and 0.2 cc. of physiological salt solution. Human thrombin, 0.1 cc., was added at once, and the coagulation and liquefaction times again recorded. By successively transferring 0.5 cc. of each digestion mixture to another tube containing fibrinogen, a titration of the active principle in the presence of increasing concentration of dissolved fibrin was accomplished.

In view of certain differences in human and rabbit fibrinogens, to be described in detail later (3), a second and similar titration was carried out in exactly the same manner with the exception that human fibrinogen was substituted for the rabbit fibrinogen. The results of the experiment are shown in Table V.

In Experiment I the test was carried out in such a manner that the fibrinolytic substance was transferred immediately after liquefaction was complete. Under these experimental conditions it may be seen (Table V) that the fibrinolytic agent is not bound as a result of the lytic action. That the fibrinolysin remains, in considerable amount, uninhibited, is evidenced by the fact that the ultimate dilution to 0.001 cc. was effective in 20 minutes. Since the filtrate used in Experiment I, when diluted to 0.0001 cc. with physiological salt solution, effected dissolution in 50 minutes, the observation just described indicates that the fibrinolysin may be present in an active state in the reaction products.

Experiment II.—A mixture of 2.0 cc. of rabbit fibrinogen, 2.0 cc. of physiological saline, and 5.0 cc. of the fibrinolytic solution employed in the above experi-

ment was coagulated by the addition of 1.0 cc. of human thrombin. At stated intervals of incubation 0.5 cc. portions of the liquefied mixture were withdrawn and added to tubes which contained 0.2 cc. of rabbit fibrinogen and 0.2 cc. of salt

TABLE V
Titration of Fibrinolysin Present in the Reaction Mixtures

<i>m</i> 1 37 4	Rabbit f	ibrinogen		Human f	ibrinogen	Resulting
Tube No.*	Coagulation time	Liquefaction time	Tube No.	Congulation time	Liquefaction time	concentration of fibrinolysin
	min.	min.		min.	min.	cc.
RΙ	1	2	ні	1	3	0.5000
RII	1	2	HII	2	2	0,2500
RIII	1	3	HIII	1	3	0.1250
R IV	1	2	H IV	1	4	0.0625
R V	1	2	ΗV	1	5	0.0318
R VI	1	3	H VI	2	5	0.0159
R VII	1	5	H VII	2	11	0.0079
R VIII	1	8	H VIII	2	30	0.0039
RIX	2	14	H IX	2	25	0.002
RX	2	20	нх	4	-	0.001

All tubes incubated in water bath at 37.5°C.

TABLE VI

Effect of Prolonged Incubation on Activity of the Fibrinolysin in the Presence of the
Liquefied Reaction Products

Duration of	Rabbit f	ibrinogen	Human fibrinogen			
incubation	Coagulation time	Liquefaction time	Coagulation time	Liquefaction time		
	min.	min.	min.			
15 min.	1	4	1	4		
30 min.	1	5	1	4		
1 hour	2	5	2	3		
2 hours	3	5	2	3		
8 hours	4	5	3	3		
18 hours	3	5	3	3		

All tubes incubated in the water bath at 37.5°C.

solution. Coagulation was induced by the addition of 0.1 cc. of human thrombin. A similar experiment was carried out with human fibrinogen. The results of the experiment are presented in Table VI.

<sup>\*</sup> Each tube received 0.5 cc. of the contents of the preceding tube; lytic test performed by adding 0.2 cc. fibrinogen, 0.2 cc. of physiological salt solution, and 0.1 cc. of human thrombin. Experiment terminated after tenth transfer.

From the table it may be seen that the fibrinolytic activity is not bound by the products resulting from the liquefaction of the fibrin clot and that exposure to the dissolved fibrin for as long as 18 hours exerted only slight inactivating effect upon the fibrinolytic principle. These facts point to the catalytic nature of the active principle, which remains free in test mixtures. Although, in experiments to be described later (3), human and rabbit fibrinogen presented certain differences, in the tests just described no essential dissimilarity was demonstrable.

Optimum Temperature for Fibrinolysis.—The optimum temperature for fibrinolytic action lies between 35° and 45°C. At temperatures of 45–55°C. a partial denaturization of the fibrinogen interferes with the clotting property and above 56°C. actual precipitation occurs. Comparative tests with material kept at ice box temperature, room temperature, and 37.5°C. have been made, and give, on the average, the following results:

In ice box: slight softening, 2 hours. At room temperature: 10-30 minutes. At 37.5°C.: 1-2 minutes.

#### DISCUSSION

The results, which comprise this report, consist of observations which define some of the properties of the fibrinolytic principle elaborated by *beta* hemolytic streptococci.

The active fibrinolysin, present in filtrates of broth cultures, has been isolated by several different procedures. The most successful methods were precipitation with three volumes of alcohol, and adsorption on alumina followed by elution with phosphate buffer. These preparations have been concentrated and evaporated to dryness. The resulting products have retained their potency for indefinite periods of time. Although the active preparations still contain impurities, the isolated lytic material is useful for purposes of standardization and will facilitate further attempts at purification.

The fibrinolysin acts, in many respects, as a relatively stable substance, retaining activity throughout the treatment to which it is subjected and resisting a considerable degree of heating as well as desiccation. Final assay of the properties is, however, rendered difficult because of the fact that the results may vary with different test materials. Some of these conditioning factors have been described; others remain unexplained.

The usual protein tests with preparations of the fibrinolysin are positive and solutions of the active material are rapidly and completely inactivated by the addition of a small quantity of trypsin or activated papain. The methods of isolation and the properties of the active principle strongly suggest that the active agent is an enzyme or catalyst which has a striking specificity for human fibrin and which induces unique physical and chemical changes. Exact chemical identification of the active substance has not yet been accomplished, but all the tests so far performed on impure material point to its protein nature.

#### SUMMARY

The active fibrinolytic principle present in cultures of hemolytic streptococci can be isolated in stable form, and partially purified by the following methods:

- 1. Precipitation of culture filtrate with 3 volumes of 95 per cent ethyl alcohol.
- 2. Adsorption upon polyaluminum hydroxide B of Willstätter (5) followed by elution with M/10 sodium phosphate buffer, pH 7.3.

Concentration can be best accomplished by vacuum dialysis (4) of either culture filtrates or preparations obtained by adsorption and elution.

The streptococcal fibrinolysin is characterized by the following properties:

- 1. It may resist heating to 100°C. for 60 minutes; variations in thermal resistance are described.
- 2. Partially purified preparations give positive tests for protein. Activity is rapidly and completely destroyed by trypsin or papain.
- 3. The active principle is demonstrable in dissolved fibrin even after 18 hours incubation.

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## BIOCHEMICAL STUDIES ON THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

#### II. NATURE OF THE REACTION

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The preceding paper (1) has described some of the properties of the fibrinolysin of hemolytic streptococci. The present report presents results which indicate the nature of the reaction and the composition of the end-products.

Although the streptococcal fibrinolysin should, in all probability, be classified as an enzyme or catalyst, its activity differs, in certain respects, from proteolytic ferments. The special characters will be demonstrated by comparative experiments in which fibrin and other substrates are subjected to fibrinolysin and other enzymes.

That the fibrinolytic principle of hemolytic streptococci induces a physical change in fibrin is self-evident by the transformation of solid matter to solution. A chemical study of the reaction presents difficulties inherent in attempts to analyze solutions composed of mixtures of proteins. Consequently, the results to be given are not final, but they indicate the qualitative changes which are involved in the phenomenon of fibrinolysis. More exact studies are now in progress and will be reported in a separate communication.

#### Materials and Methods

The methods of obtaining the fibrinolytic substance from hemolytic streptococci and of preparing fibrinogen and thrombin solutions were described in previous articles (1, 2). It is important to note, however, that human materials have been almost exclusively used in the experiments to be reported. Since the specificity of

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the fibrinolysin for human fibrin is so striking and since this study centers around the reaction products, the necessity of employing human material is apparent.

Where rabbit fibrinogen is employed, special mention of the fact is made.

Other technical procedures are given in the text according to the type of experiment which was performed.

#### Action of the Fibrinolytic Principle upon Various Substrates

Before proceeding with a description of analytical experiments concerning the effect of fibrinolysin on fibrin, it is desirable to orient the active bacterial substance with respect to other ferments and other substrates. The purpose of each experiment will become apparent from the substrate which was selected and the enzyme which was employed for comparison.

1. Effect of Fibrinolysin and Trypsin on Casein and Gelatin.—These experiments were designed to test the proteolytic properties of the fibrinolytic principle. Casein and gelatin were selected as appropriate substrates, and the experiments were controlled with parallel determinations in which trypsin was substituted for the fibrinolytic principle.

Preparation of Materials.—A 1.25 per cent solution of Bacto gelatin in buffered physiological saline, pH 7.4, was used as a gelatin substrate. A similar solution was prepared from commercial casein which had been purified once by the method of Northrop (3).

The trypsin solution contained 5 mg. of a commercial product per cc. of physiological salt solution.

The fibrinolytic solution was prepared by dissolving 500 mg. of a dry, alcohol-precipitated material in 50 cc. of salt solution. The usual test proved this solution to have a fibrinolytic titer of 0.001 cc. in 34 minutes.

Procedure.—Duplicate 40 cc. portions of the casein substrate were treated with 10 cc. of the trypsin solution and 10 cc. of the fibrinolytic preparation respectively. Two flasks containing 40 cc. of gelatin substrate were similarly subjected to trypsin and fibrinolysin. Control solutions containing one only of each of the various components of the protocol were prepared. Both the test solutions and the controls were incubated at 37.5°C. Toluene was used as a preservative. Analyses were carried out at once and after 24 and 48 hours incubation.

Analytical Method.—A 5 cc. portion of each digestion mixture was used for each analysis. The accurately measured sample was placed in the deaminizing bulb of a Van Slyke macro amino nitrogen apparatus and deaminization was permitted to proceed for 15 minutes. The evolved nitrogen was collected and measured in a micro burette. Duplicate determinations were made upon each solution and the

values usually agreed within 0.02 ml. of evolved nitrogen. The results of the analyses, appropriately corrected for the blank error and the amino nitrogen content of the trypsin and fibrinolytic solutions, are presented in Table I and in Text-fig. 1.

From Table I and Text-fig. 1, it is evident that, by the analytical method used, the fibrinolytic substance exerted no demonstrable hydrolytic action upon either of the two proteins, casein or gelatin. The possibility of a superficial hydrolysis, not detectable by the analytical method employed, seems improbable from the fact that a 2 per

TABLE I

Comparison of the Action of Fibrinolysin and Trypsin upon Casein and Gelatin

Preparation	Duration of	Amino N p	Amino N per 100 cc.*		
1 reparation	incubation	Found	Increase		
		mg.	mg.		
Casein + trypsin	None	3.27	_		
Casein + trypsin	24 hours	15.10	11.83		
Casein + trypsin	48 hours	17.04	13.77		
Casein + fibrinolysin	None	3.24			
Casein + fibrinolysin	24 hours	3.20	-0.04		
Casein + fibrinolysin	48 hours	3.15	-0.09		
Gelatin + trypsin	None	3.52			
Gelatin + trypsin	24 hours	19.19	15.67		
Gelatin + trypsin	48 hours	21.71	18.19		
Gelatin + fibrinolysin		3.52	-		
Gelatin + fibrinolysin		3.67	0.15		
Gelatin + fibrinolysin	48 hours	3.79	0.27		

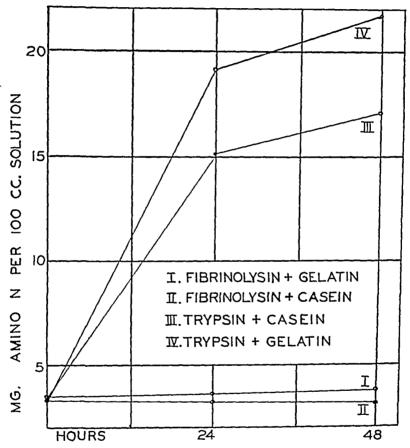
<sup>\*</sup> All values corrected for blank error and for amino nitrogen content of the trypsin and fibrinolysin solutions, respectively.

cent gelatin solution which contained 10 mg. of the fibrinolytic substance per cc. promptly solidified when it was placed in an ice bath after incubation for 72 hours at 37.5°C.

2. Effect of Fibrinolysin and Peptase (Stevens and West) on Peptone.—Stevens and West (4) have reported the presence of a proteolytic enzyme in the cell extracts of hemolytic streptococci. This enzyme was shown to attack casein, but maximal hydrolysis was observed when a peptone substrate was employed. The term peptase was therefore applied to the enzyme. The following experiment was designed to

demonstrate the difference between the fibrinolytic substance and the streptococcus peptase of Stevens and West.

Preparation of Materials. Peptase.—The sedimented cells from 3 liters of broth culture were thoroughly washed with physiological salt solution and suspended in 5 cc. of sterile distilled water. The suspension was frozen and subjected to pres-



Text-Fig. 1. Comparison of the action of fibrinolysin and trypsin upon casein and gelatin.

sure in the apparatus described by Johlin and Avery (5). The resulting mixture was warmed to room temperature, diluted to a volume of 15 cc. with physiological saline, and shaken for 15 minutes to insure complete emulsification. The solution was then centrifuged at a high speed for an hour. The supernatant fluid was

<sup>&</sup>lt;sup>1</sup> The authors are indebted to Dr. R. C. Avery of the Department of Bacteriology, Vanderbilt University, who kindly supplied the extraction apparatus.

reserved and the sediment was again frozen and subjected to disintegration by pressure. The mixture was diluted, shaken, and again centrifuged at a high speed. By microscopical examination of stained smears, most of the bacterial cells were found to be ruptured. The combined extracts, 25 cc. in volume, were mixed with a little toluene and permitted to stand in the ice box for 12 hours. The solution was divided into two parts, one of which was heated at 62°C. for 1 hour.

The fibrinolytic preparation used for comparison with the peptase was prepared by the method of adsorption and elution, just described. The eluate titred to 0.001 cc. in 14 minutes. A portion of the preparation was heated at 62°C. for 1 hour.

Procedure.—To four 10 cc. portions of a 1 per cent solution of Merck peptone were added respectively 5 cc. of the peptase solution (cell extract), 5 cc. of the fibrinolytic solution, 5 cc. of the heated peptase, and 5 cc. of the heated fibrinolytic preparation. Appropriate controls of the peptone and other solutions were

TABLE II a

Comparison of the Action of Fibrinolysin and Streptococcus Peptase upon Peptone

Preparation	Amino N per 100 cc.*			
repration	Found	Increase		
	rst.	mg.		
Peptone alone	28.82			
Peptone + fibrinolysin	28.87	0.05		
Peptone + heated fibrinolysin	28.85	0.03		
Peptone + streptococcus peptase	44.36	15.54		
Peptone + heated streptococcus peptase	28.65	-0.17		

<sup>\*</sup> All values corrected for blank error and for amino nitrogen content of fibrinolysin and peptase solutions, respectively.

prepared. A portion was withdrawn from each tube for immediate analysis. The remainder of the solutions were layered with a little toluene and incubated at 37.5°C. Samples were withdrawn for analysis after 24 to 48 hours incubation.

Analytical Method.—Analyses were carried out upon 2 cc. portions of each solution. The samples were deaminized for 15 minutes in the micro Van Slyke amino nitrogen apparatus and the evolved nitrogen collected and measured in the micro burette. Duplicate determinations upon each solution agreed within 0.02 ml. of evolved nitrogen. The results of the experiment are shown in Table II a.

From Table II a it is evident that the fibrinolytic principle is not capable of hydrolyzing peptone, whereas the cell extract exerted an intensive peptolytic action upon the substrate. It is also evident from the table that heating the peptase at 62°C. for 1 hour destroyed its enzymatic action for peptone.

A comparison of the fibrinolytic titers of the fibrinolysin and peptase solutions was carried out and the results are recorded in Table II b.

From Table II b it can be seen that the fibrinolytic solution possessed a uniformly high titer which was unaltered by exposure to a temperature of 62°C. The cell extract, on the other hand, contained only a comparatively small amount of fibrinolytic active material which probably represents the amount of intracellular fibrinolysin. The fact that exposure to a temperature of 62°C. for an hour diminished the small amount of fibrinolytic activity in the cell extract may have been

TABLE II b

Comparison of the Fibrinolytic Action of Fibrinolysin and Streptococcus Peptase

Preparation		Amount of preparation							
1 reparation	0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.	0.001 cc.			
	min.	min.	min.	min.	min.	min.			
Fibrinolysin	*3	3	6	10	12	14			
Heated fibrinolysin	3	3	7	14	14	14			
Streptococcus peptase		24	20	35	70				
Heated streptococcus peptase	120	120	90	_	_				

All tubes incubated in water bath at 37.5°C.

due to an adsorption of the fibrinolytic principle upon the extraneous heat-denatured protein material in the heated extract.

The results of the experiment are considered to justify the conclusion that the fibrinolytic activity of streptococci is referable to an active substance, chemically distinct from the peptolytic enzyme previously isolated from streptococci by Stevens and West.

3. Effect of Fibrinolysin, Trypsin, and Peptase on Fibrin.—The negative character of the above experiments indicates that the fibrinolytic principle does not possess a broad capacity to act upon proteins in general or upon peptone. That fibrin is affected is self-evident from the physical change which the clot undergoes. The nature of the chemical change in fibrin is indicated in experiments to follow. In

<sup>\*</sup> Figures represent time in minutes of interval between coagulation and complete liquefaction; the symbol (-) indicates that no liquefaction occurred in 2 hours.

order to bring out the special qualities of the fibrinolysin, trypsin and streptococcal peptase have been employed in comparable experiments.

Preparation of the Fibrin.—Potassium oxalate was employed as an anticoagulant. Human plasma was diluted with five times its volume of physiological salt solution and coagulation was induced by the addition of an excess of CaCl<sub>2</sub>. The coagulum was permitted to stand several hours to insure complete formation of the fibrin. By careful manipulation of a stirring rod the liquid was pressed from the gel and the fibrin rolled into a compact mass. This product was washed thoroughly with distilled water, pressed dry, and placed in a vacuum desiccator over phosphoric anhydride. The resulting brittle product was ground to a fine white

TABLE III

Comparison of the Action of Fibrinolysin, Streptococcus Peplase, and Trypsin upon

Human Fibrin

	Amino nitrogen per 100 cc.º					
Preparation	24 hrs.	Increase	72 hrs.	Total increase		
	r:z.	rig.	r:s.	FIG.		
Fibrin alone	7.37	_	7.89	0.52		
Fibrin + fibrinolysin	13.18	5.81	18.33	10.96		
Fibrin + heated fibrinolysin†	8.57	1.20	9.55	2.18		
Fibrin + streptococcus peptase	26.40	19.03	25.78	18.41		
Fibrin + heated streptococcus peptase		2.13	9.98	2.61		
Fibrin + trypsin	37.79	30.22	43.83	36.46		

<sup>\*</sup> All values corrected for blank error and for the amino nitrogen content of fibrinolysin, streptococcus peptase, and trypsin solutions, respectively.

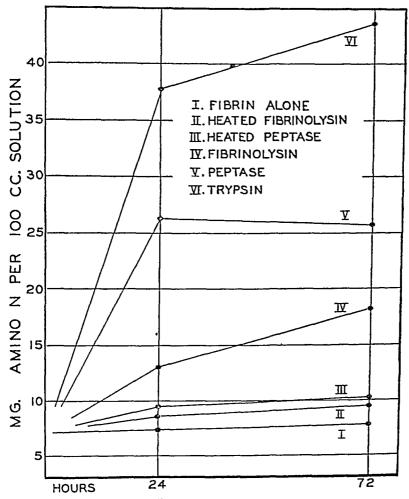
powder in a mortar. The combined and uniformly mixed fibrin from several preparations was utilized in the following experiment.

Active Solutions.—A solution of commercial trypsin was made up in a concentration of 4 mg. per cc. of physiological salt solution. A streptococcal peptase solution was prepared in the manner just described. The peptase solution, 25 cc. in volume, contained the material extracted from the sedimented cells from 4 liters of broth culture. The solution was divided and one portion was heated at 60°C. for 1 hour. The fibrinolytic solution contained 10 mg. of an alcohol-precipitated preparation per cc. of salt solution. The titer of this solution was 0.001 cc. in 43 minutes. A portion of the solution was heated for an hour at 60°C.

Precedure. - Duplicate series of small flasks were prepared, each of which con-

<sup>†</sup> The fibrinolysin employed in this experiment had been precipitated with alcohol and was therefore heat-labile.

tained 200 mg. of the powdered fibrin and 15 cc. of buffered saline, pH 7.2. Accurately measured 5 cc. portions of the various test solutions were added to the substrate suspensions, and the mixtures, together with appropriate controls, were placed in the incubator at 37.5°C. Toluene, 1 cc., was added to each flask to insure sterility. One group of flasks was removed after 24 hours incubation, the con-



TEXT-Fig. 2. Comparison of the action of fibrinolysin, streptococcus peptase, and trypsin upon human fibrin.

tents carefully filtered, and amino nitrogen determinations carried out upon aliquot portions of the filtrate. The second series of flasks were removed from the incubator after 72 hours and the contents similarly treated. The details of the analytical method were the same as those described in the experiments with casein and gelatin. The results of these analyses are presented in Table III and in Text-fig. 2.

The values recorded in Table III and Text-fig. 2 present an interesting contrast to the results of experiments with the casein, gelatin, and peptone substrates. Trypsin hydrolyzed the fibrin with a sharp increase in the amino N according to the well recognized proteolytic action of this ferment. The fact that the peptase possesses the property of hydrolyzing fibrin is of interest in view of the results of Stevens and West who found this enzyme incapable of hydrolyzing either serum or serum albumin.

Of more immediate interest to the present purpose, however, is the fact that, at the end of 72 hours incubation, a not inconsiderable amount of amino nitrogen was demonstrated to be present in the fibrin-fibrinolytic principle mixture. A correct evaluation of this increase is not yet possible. Two interpretations may be considered. The results may signify that the fibrinolytic principle is comparable to a proteolytic enzyme which acts specifically in promoting the hydrolysis of human fibrin. On the other hand, a more probable explanation lies in the dissolving action of the fibrinolytic substance upon the insoluble fibrin substrate. Under this condition the increase in the amino nitrogen may be accounted for by an increased protein concentration of the solution. From this standpoint it is perhaps significant that the increase in amino nitrogen in the mixtures took place at a more gradual and constant rate than was observed with either trypsin or the peptase. Furthermore, this latter view, which implies that the chemical structure of fibrin is not extensively altered by the process of dissolution, receives additional support from the fact that the end-product of the reaction acts, in other tests, as a protein. A study of the mechanism of the reaction is being continued by experimental procedures which, it is hoped, will leave no question with regard to the rôle of hydrolysis in the dissolution of the fibrin.

4. Effect of Fibrinolysin on Fibrinogen.—In this and previous reports emphasis has been placed upon the fact that the fibrinolytic principle acts specifically upon clotted human fibrin and fails to act on rabbit fibrin when composed entirely of rabbit constituents. It became of interest to determine the activity of fibrinolysin upon the two species of fibrinogens, precursors of fibrin. The technique of the experiments and the results are as follows.

Procedure.—Human fibrinogen, 4 cc., was diluted with 4 cc. of physiological salt solution. A 10 cc. portion of an adsorbed, eluted, and concentrated fibrinolytic preparation was added and the mixture placed in the 37.5°C. water bath. A similar solution containing rabbit fibrinogen and the fibrinolytic solution was prepared and incubated simultaneously. At frequent intervals, during incubation, 0.9 cc. samples of each solution were withdrawn, and mixed with 0.1 cc. portions of human thrombin. The coagulation time and the interval required for liquefaction of the clots were recorded. The results are shown in Table IV.

The table shows that when fibrinolysin and human fibrinogen are incubated for 20 minutes, the clotting power of the fibrinogen is seri-

TABLE IV

Comparison of the Action of Fibrinolysin upon Human and Rabbit Fibrinogen

Duration of incuba- tion of fibringen	Human i	ibrinogen	Rabbit fibrinogen		
with fibrinolysin	Coagulation time   Liquefaction time		Coagulation time	Liquefaction time	
	min.	min.	min.	min.	
None	*1	7	1	6	
• 5 minutes	2	6	2	4	
10 minutes	2	5	2	4	
20 minutes	†2	5	1	4	
35 minutes	· 	_	1	4	
50 minutes			2	3	
5 hours	. —		2	4	
18 hours	<del></del>		2	5	

All tubes incubated in water bath at 37.5°C.

ously impaired, and that after 35 minutes incubation, the fibrinogen no longer is capable of forming fibrin clot. In contrast to the change induced in the human protein, rabbit fibrinogen, even after 18 hours exposure to fibrinolysin, remains unchanged and promptly solidifies when thrombin is added. This resistance of rabbit fibrinogen presents an interesting biochemical difference between the two species of fibrinogens and is undoubtedly an important factor in determining the insusceptibility of rabbit fibrin to the fibrinolytic action.

<sup>\*</sup> Figures represent time in minutes; the symbol (-) indicates no coagulation occurred in 2 hours.

<sup>†</sup> Clot imperfectly formed.

#### The Protein Nature of the Reaction Products

The experiments to be described consist of a comparison of the well defined properties of fibrinogen solutions with those of solutions containing the products of fibrinolysis and solutions of fibrinogen which have been exposed to and altered by fibrinolysin.

Human fibrinogen is a salt-soluble, water-insoluble globulin, precipitable by 50 per cent saturation with sodium chloride, or 25 per cent saturation with ammonium sulfate. It is denatured by exposure to a temperature of 57°C. It is particularly characterized by its transformation into the insoluble fibrin through the agency of thrombin.

A study of these four properties of fibrinogen have, therefore, been applied to the products of fibrinolysis and to fibrinogen-fibrinolysin mixtures. In these experiments, materials derived from human sources have been exclusively employed.

- 1. Precipitation with NaCl.—When NaCl is added up to 50 per cent saturation to the products of fibrinolysis no precipitate or clouding occurs. Similarly after fibrinogen-fibrinolysin mixtures have been incubated for 60 minutes, 50 per cent NaCl saturation exerts no precipitating effect.
- 2. Precipitation with  $(NH_4)_2SO_4$ .—When  $(NH_4)_2SO_4$  is added to the products of fibrinolysis in amounts sufficient for 25 per cent saturation, no precipitation occurs. The same negative result is obtained with incubated fibrinogen-fibrinolysin mixtures.

When, however, in the products of fibrinolysis or fibrinogen-fibrinolysin mixtures the saturation of  $(NH_4)_2SO_4$  is raised to 35 per cent, a large yield of precipitate is obtained. Furthermore, by increasing the saturation to 40 and 45 per cent additional flocculation occurs. These results are of interest since the fibrin after dissolution, and the fibrinogen-fibrinolysin mixtures after incubation, are abundantly precipitated by the per cent saturations of  $(NH_4)_2SO_4$  which flocculate serum globulins.

- 3. Effect of Heat.—Fibrinogen, the products of fibrinolysis, and fibrinogen-fibrinolysin mixtures were incubated at 37.5°C. for 1 hour. Each preparation was then heated at 57°C. for 30 minutes. The untreated fibrinogen flocculated abundantly; in the other two preparations, only a moderate cloud and a slight precipitation occurred. Heating the latter two solutions at 75°C. caused a heavy precipitation. Quantitative studies are now in progress to define more exactly the differences demonstrable in these experiments with different temperatures.
- 4. Addition of Thrombin.—Untreated fibrinogen, the products of fibrinolysis, and fibrinogen-fibrinolysin mixtures were incubated at 37.5°C. At intervals of 5, 10, 15, and 30 minutes, samples of each were removed and mixed with thrombin. With the fibrinogen, clotting took place promptly with each specimen. With

the products of fibrinolysis, the addition of thrombin was entirely without effect. With the fibrinogen-fibrinolysin mixture, clotting occurred only in the 5 minute samples; in the 10 minute sample a few strands of fibrin appeared; in the remaining test no coagulation occurred.

The qualitative character of the products resulting from the action of streptococcal fibrinolysin upon fibrin and fibrinogen is shown by the experiments just described. The results indicate that, although the changes are distinct, the end-product still has properties of protein, which is apparently newly formed. The results further substantiate the opinion that the dissolution of the fibrin is not accompanied by extensive hydrolysis.

Investigations of a more exact identification of the end-product are now in progress.

#### DISCUSSION

A comparison of the action of streptococcal fibrinolysin and other enzymes on fibrin and other substrates, demonstrates certain unique biochemical properties of the bacterial product.

The specificity of the fibrinolysin is particularly interesting. Differences in action on human and animal clot have been previously reported (1, 6). The insusceptibility of rabbit plasma clot finds a probable explanation in the fact that rabbit fibrinogen is not altered by the fibrinolysin. The basis of the resistance of rabbit material in contrast to the susceptibility of human protein seems to depend upon a difference in the chemical constitution of the two substances even though they both are physiologically precursors of fibrin.

Additional evidence of the specificity is brought out by the apparent incapacity of the fibrinolysin to attack casein, gelatin, or peptone. The fibrinolytic principle does not act upon proteins in general and its limitations are strikingly exemplified by contrast to trypsin. Of equal interest is the sharp difference between peptase and fibrinolysin both derived from hemolytic streptococci. The peptase of Stevens and West is an intracellular proteolytic enzyme especially potent against peptone. The fibrinolysin is a product of the same cultures but is chiefly extracellular and possesses different capacities.

At the present time the experimental data have not clearly defined the course of the fibrinolytic reaction. Whether or not the dissolution is accompanied by definite proteolytic hydrolysis is uncertain, and awaits further study. However, the fact that the end-products, even after 72 hours incubation, still have the characteristics of a protein, indicates the unusual effect exerted by the streptococcal fibrinolysin. The gradual and constant increase in the amino nitrogen content of a mixture containing the fibrinolytic principle and human fibrin suggests that, in addition to the dissolution of the insoluble substrate, an unexplained and perhaps very slight splitting of the molecule has taken place. It would be premature, at the present time, to speculate concerning the correct interpretation. Studies now in progress will attempt to define more accurately the mechanism involved in the fibrinolytic phenomenon.

#### SUMMARY

The fibrinolysin of hemolytic streptococci exerts no hydrolytic action upon casein, gelatin, or peptone.

The action on solid human fibrin is characterized by a small and gradual increase in the amino nitrogen content of the solution.

The specific and special enzymatic action of fibrinolysin is contrasted with trypsin and with streptococcal peptase (4).

Solutions of human fibrinogen, after brief incubation with fibrinolysin, lose the capacity to form fibrin. Solutions of rabbit fibrinogen, on the other hand, retain the property of transformation into fibrin, even after prolonged exposure to fibrinolysin.

Qualitative tests, with solutions resulting from the action of streptococcal fibrinolysin on human fibrin, indicate that the end-product may be protein and that the degradation of the molecule is not great.

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#### THE FATE OF AVIRULENT HEMOLYTIC STREPTOCOCCI INJECTED INTO THE SKIN OF NORMAL AND SENSITIZED RABBITS

#### LOCAL FIXATION OF BACTERIA

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The present study was undertaken to determine the fate of bacteria introduced into the skin of sensitized animals, but it was soon found that the behavior of bacteria in the normal skin was not clearly understood. Many observations have shown that an inflammatory reaction prevents the spread of bacteria, particulate matter, and certain dyes. The relationship of sensitization and immunity to local inflammation and to the fixation of bacteria is still obscure.

In 1906 Noetzel (1) demonstrated that B. pyocyancus if injected into the knee joint of rabbits can be demonstrated within 5 minutes in large numbers in the inguinal, crural, and lumbar lymph nodes and also in the blood stream, liver, kidney, and spleen. Pawlowsky (2) repeated these observations and in addition found that if staphylococci are introduced into the knee joint of a guinea pig, they may be demonstrated in the blood stream and organs in from 24 to 48 hours after injection. If a sterile irritant such as alcohol or turpentine is introduced so as to produce an inflammation, and later the staphylococci are injected, there is no spread to the blood stream.

Gay and Morrison (3) working on experimental empyema have shown that if an irritant such as meat infusion broth or egg white be introduced into the pleural cavity of a rabbit, many times the minimal lethal dose of streptococci can be injected into the pleural cavity 24 hours after the onset of inflammation without causing the death of the animal.

Opie (4) showed that when streptococci are injected into the peritoneal cavity of a normal rabbit, they reach the blood in large numbers with great rapidity, but if a sterile irritant such as aleuronat is introduced prior to the bacteria, the dissemination from the peritoneal cavity is delayed. After the infiammation has been present for 48 hours before the injection of bacteria, there is almost complete protection.

In 1924 Opie (5) demonstrated that a rabbit sensitized by several intradermal injections of foreign protein (horse serum or egg white) will fix this protein at the site of injection and prevent dissemination into the blood stream. The same protein injected into the skin of a normal rabbit will rapidly leave the site of injection and enter the blood stream. He attributed the fact that the protein remained fixed in the sensitized animal, to the inflammation resulting from a meeting of antigen and antibody at the site of injection.

Willis (6) studied the spread of tubercle bacilli from the site of injection in normal guinea pigs and in animals previously infected with living avirulent tubercle bacilli. By removing the tissue at the site of inoculation 1 hour after injection, he was unable to prevent a generalized tuberculous infection in normal animals, whereas if he removed it from the immunized animal within 4 days he was able to prevent the development of generalized tuberculosis. He determined the presence of tubercle bacilli in the adjacent lymph node by inoculating the tissue into normal guinea pigs. It was found that the bacilli reached the node of a normal animal within 24 hours, whereas they did not appear in the node of the immune animal for 2 to 3 weeks. Freund (7) has recently been able to obtain similar results by using guinea pigs immunized with heat-killed tubercle bacilli until they showed hypersensitiveness to old tuberculin. After injection with tubercle bacilli the animals were killed at short intervals. Tubercle bacilli were grown from the lymph nodes of the control animal 1 day after infection, whereas the nodes from the vaccinated animals did not show bacteria until much later.

Menkin has recently reported numerous experiments demonstrating the fixation of various dyes and bacteria (8) in inflamed areas and has written an excellent review (9) of the entire subject.

Cannon and Pacheco (10) immunized a group of guinea pigs with a staphylococcus vaccine by repeated injections into a circumscribed area of the skin over the abdomen. They then infected these animals with a virulent culture of Staphylococcus aureus injected intradermally into the same site. A group of normal guinea pigs was infected with the same dosage at the same time. The normal animals showed a diffuse cellulitis of the abdominal wall and frequently died within 24 hours. Those that had been immunized showed a local area of suppuration that ulcerated and healed with no serious consequence. In the normal animal the organisms were diffusely scattered throughout the skin, whereas in the immunized animal they were gathered in clumps and showed a definite tendency to localization at the site of injection. These workers have attributed this localization to what they term a tissue immunity.

These observations establish the fact that there is some mechanism whereby bacteria in an inflamed area are prevented from spreading into the surrounding tissues. Menkin believes that deposition of fibrin in tissue spaces and mechanical blockage of lymphatics are important factors in this local fixation.

Animals immunized against tubercle bacilli evidently prevent dissemination of tubercle bacilli to the regional lymphatics. It has been suggested that the bacilli do not disseminate because they are destroyed by the immune animal shortly after injection. It is important to know whether dissemination is prevented by destruction or by local fixation of bacteria, perhaps with subsequent multiplication of the organisms.

Although several observers (Buxton and Torrey (11), Bull (12)) have studied the fate of bacteria injected into the serous cavities or have studied their rate of disappearance from the blood stream, there is little information concerning the dissemination of bacteria introduced into the skin of normal rabbits. Do changes at the site of injection or within lymph nodes of normal or immune animals effectively prevent their penetration into the blood stream and internal organs?

#### Methods

The organism used in the following experiments was a relatively non-virulent hemolytic streptococcus obtained from a case of peritonitis. An 18 hour culture grown in fresh rabbit's blood broth was used. The organism grown in this media for a period of 18 months has shown no tendency to clump and still produces lesions in the skin of rabbits similar to those observed 1 year ago.

In order to determine the number of streptococci injected into and recovered from the skin of a rabbit, a suitable method for counting bacteria was essential. The plating method discredited by some observers is not entirely accurate, but if the culture is thoroughly shaken with glass beads and then mixed with a pipette, there are many single cocci and the chains in the first dilution average from 3 to 5 cocci per chain, whereas in the undiluted culture the chains are much longer and there are fewer single cocci.

In the earlier experiments 0.1 cc. of the culture to be counted was diluted 1:100, 1:1,000, and 1:10,000. These dilutions were made in tubes of uniform size each containing three glass beads. After mixing with a pipette, the tubes were thoroughly shaken. 0.1 cc. of each of the above dilutions was transferred to a tube of melted agar to which 0.5 cc. of defibrinated rabbit's blood had been added. The contents were shaken and poured in a Petri dish. Two plates were usually poured from each dilution. As a general rule the number of colonies in the duplicate plates checked fairly well, but since there was occasionally rather wide variation, some more accurate method was sought.

The method used by Wilson (13) was tried. Tubes measuring  $150 \times 16$  mm, each containing 2.5 cc. of agar were used. The agar was melted and when it was cooled to  $50^{\circ}\text{C}_{\odot}$ , 4 drops of defibrinated rabbit's blood and 0.1 cc. of the dilution

to be cultured were added. The tube was well shaken and, held horizontally, was rolled under a cold water tap until the contents solidified in a thin layer adherent to the glass. The tubes were incubated upside down and the colonies counted after 48 hours. With this method the counts were found to check more closely than with the plate method. The following counts are representative of those obtained during the course of the experiments.

Culture diluted	1:70,000	1:700,000
1. No. of organisms per tube	497	53
« «   «   «   «	460	26
Average No. of organisms per tube	478	39
Calculated No. of organisms in 0.1 cc. 1:70 dilution	478,000	390,000
2. No. of organisms per tube	195	43
« « « « «	334	27
Average No. of organisms per tube	264	35
Calculated No. of organisms in 0.1 cc. 1:70 dilution	264,000	350,000

When streptococci are present in a suspension in great numbers, counts have occasionally varied within wide limits and it cannot be claimed that the method measures the actual number of streptococci present in the skin. Nevertheless the figures given in the tables are roughly comparable and as the experiments will show, the method is sufficiently accurate to determine the fate of streptococci injected into the skin.

#### Fate of Streptococci in Normal Rabbits

Experiments were performed to determine the fate of hemolytic streptococci injected in different quantities into the skin of normal rabbits.

The site chosen for injection was the right flank about 4 cm. anterior to the right inguinal lymph node, which is readily palpated through the skin of a normal animal. A superficial vein in this area runs to the lymph node and serves as a rather constant landmark for injection. Trypan blue in saline solution injected into the skin in this area will rapidly and regularly accumulate in this lymph node, but if the injection is made too far anteriorly, a part of the dye will reach the axillary nodes. Cultures were made from the subcutaneous inguinal lymph node and in several instances from the next node in the chain of lymphatics, which is a small node usually found at the bifurcation of the external and internal iliac vessels. Considerable care was required in removing this node on account of its close association with the blood vessels.

The first two rabbits listed in Table I received 0.1 cc. of a broth culture of hemolytic streptococcus and the rest received a smaller dose, namely 0.1 cc. of a 1:10 dilution of a similar culture. The rabbits were killed at intervals varying from 15 minutes up to 100 hours. The skin was cleaned with alcohol and distilled water. With aseptic technique an anteroposterior incision about 5 cm. long was made over the site of the inguinal lymph node, which was easily removed with sterile forceps and scissors and transferred to a sterile container. The tissue at the site of injection was next removed as a piece of skin measuring about  $4 \times 4$  cm. which was transferred to another sterile container.

The abdomen was now opened and the right iliac lymph node removed. A blood culture was made from the heart of all the rabbits.

The skin pinned taut on sterile gauze with the epidermal surface down, was carefully scraped with a sharp scalpel. The material obtained was thoroughly ground in a mortar and 9.9 cc. of broth were slowly added, making a 1:100 dilution; 1 cc. of this was added to 9 cc. of broth. Dilutions were made in this manner up to 1:100,000. 0.1 cc. of 1:10,000 and 1:100,000 dilutions were plated in blood agar.

The lymph nodes were ground in a mortar and 2 cc. of broth were added drop by drop; 1 cc. of the suspension was mixed with melted agar to which rabbit's blood had been added, and plated.

After the cultures had been incubated for 48 hours, the colonies were counted. Though cultures showing no growth were kept for 1 week, a colony seldom developed after the first 2 days of incubation. Only colonies showing a definite zone of hemolysis were counted. Smears were made of doubtful colonies and they were subcultured on blood agar and broth to check hemolysis and chain formation.

To determine if the time elapsing between death of the animal and preparation of cultures was sufficient to permit any significant multiplication of bacteria in the excised skin, the following experiment was performed.

A normal rabbit was injected intracutaneously at 3 sites with 0.1 cc. of a 1:70 dilution of hemolytic streptococci. It was killed immediately and the 3 pieces of excised skin were left in sterile dishes at room temperature. Cultures were made at intervals of  $\frac{1}{2}$ , 1, and  $1\frac{1}{2}$  hours.

	6,000
No of highest and accounted from this often 1 has at some temporaries 1	-13
No, or pacteria recovered from skin after 4 nr. at room temperature)	5,000
No. of bacteria recovered from skin after 1 hr. at room temperature. 4	0.000
No. of bacteria recovered from skin after 11 hrs. at room temperature. 2	0,099

These figures indicate that there is little multiplication in the skin at room temperature during 1½ hours.

Site of Injection.—When 0.1 cc. undiluted or 0.1 cc. of a 1:10 dilution of hemolytic streptococci was injected into the skin of normal rabbits, fewer organisms were recovered than were injected in all but one instance (R. 70, Table I), and here the difference was slight. On the other hand, when a smaller dose (0.1 cc. 1:70) was injected intradermally into normal animals (Table II) more organisms were recovered than injected in about half of the rabbits. The multiplication began

TABLE I

Streptococci in the Skin, Lymph Nodes, and Blood of Normal Rabbits after the
Intracutaneous Injection of the Organism

Davisons			No. of	No. of s	treptoco overed	cci			
Designa- tion of rabbit	Time interval			Skin	Ingui- nal lymph node	himph	bloo	Results of blood culture	
R. 40	2 hrs.	0.1 cc. undiluted	6,430,000	9,514,000	211	25	No	growth	
R. 39	4 "	0.1 " "	6,730,000	618,000	76	101	"	"	
R. 38	15 min.	0.1 cc. 1:10	736,000	135,000	0	0	"	"	
R. 57	15 "	0,1 " 1:10	772,000	590,000	1,940	40	11	46	
R. 55	15 "	0.1 " 1:10	320,000	34,000	50	6	"	"	
R. 42	30 "	0.1 " 1:10	1,808,000	34,000	25	0	"	"	
R. 70	34 "	0.1 " 1:10	139,000	165,000	1	0	"	"	
R. 12	50 "	0.1 " 1:10	630,000	68,000	35	0	"	"	
R. 47	1 hr.	0.1 " 1:10	333,000	98,000	2,447	2	"	"	
R. 56	2 hrs.	0.1 " 1:10	320,000	318,000	0	0	"	"	
R. 69	2 "	0.1 " 1:10	692,000	213,000	2	0	**	"	
R. 46	8 "	0.1 " 1:10	333,000	140,000	0	0	"	"	
R. 41	24 "	0.1 " 1:10	1,808,000	307,000	178	370	u	"	
R. 62	48 "	0.1 " 1:10	772,000	1 colony	0	0	"	**	
R. 63	100 "	0.1 " 1:10	772,000	0	0	0			

within 30 minutes, became conspicuous after the 1st hour, reached a maximum in from 3 to 5 hours, and then rapidly subsided.

Nevertheless in two instances, R. 41 (Table I) and Experiment 23 (Table II), the infection failed to subside. After 24 hours great multiplication of streptococci had occurred and up to the time when the animal was killed, the infection was progressive. The conditions that caused this unfavorable course were not evident.

To determine if hemolytic streptococci can multiply in the skin of a rabbit as rapidly as these observations made during the first 5 hours after inoculation indicate, the experiment that follows was performed.

0.1 cc. of a 1:70 dilution in broth of an 18 hour culture of hemolytic streptococci was injected into the skin at 2 sites in the flank of a rabbit killed immediately before injection. One piece of skin was removed immediately and cultured as in the experiments on living animals. The rabbit was then placed in an incubator at 37°C. for 2 hours, when the remaining site of injection was removed and cultured. The results are as follows:

No. of bacteria injected	220,000					
No. of bacteria recovered immediately after injection	83,000					
No. of bacteria recovered after incubation of 2 hrs	3,550,000					
	•					

Bacteria in the skin of a recently killed rabbit where they are not destroyed multiply at body temperature with great rapidity, in this instance about 17 times.

Inguinal Lymph Node.—When the larger dose, 0.1 cc. undiluted, or 0.1 cc. 1:10 of streptococci (Table I) was injected, we recovered organisms from the inguinal lymph nodes in fairly large numbers during the 1st hour. After this period very few organisms were recovered. Streptococci reached the inguinal lymph node within 15 minutes in 2 of 3 rabbits. They were recovered from the inguinal lymph node in 10 of the 15 rabbits (66.6 per cent).

It is noteworthy that R. 40 and R. 39, in which after 2 and 4 hours streptococci appeared in the lymph nodes, received an unusually large number of bacteria.

With the smaller dose, namely 0.1 cc. of a 1:70 dilution (Table II), the passage of organisms to the lymph nodes continued during 7 hours and then almost completely ceased coincidentally with their disappearance from the skin.

In 2 animals, R. 41 (Table I) and the normal rabbit in Experiment 23 (Table II), mentioned above as exceptions to the usual course of infection, streptococci were found in lymph nodes later than 8 hours following injection. In both of these animals, continued penetration into the lymph nodes had been accompanied by unusual multiplication of organisms at the site of injection.

Iliac Lymph Node.-Bacteria were recovered from the iliac lymph

node in moderate numbers in 6 of 15 animals that had received the larger dose of organisms (Table I), and in small numbers from 2 of animals that received the smaller dose of 0.1 cc. 1:70 (Table II) They were present in this node in those animals that had large number of streptococci in the inguinal lymph nodes. It cannot be assumed that all of the lymph that reaches the iliac node has passed through the inguinal, but cultural differences after inoculation give some in sight into the relation of proximal and distal nodes to skin infection and show that these nodes are fairly effective bacterial filters, though they permit the passage of some bacteria.

It is noteworthy that the passage of streptococci from inguinal to iliac lymph nodes occurred in great part within the first 15 minutes following intracutaneous injection. In the one instance (R. 47) in which colonies were obtained from the iliac nodes after 1 hour, there was an unusually large number in the inguinal node. In the one instance (R. 41) in which there were streptococci in the iliac lymph node after 24 hours, there was progressive infection involving both skin and lymph nodes.

Blood and Internal Organs.—Organisms were recovered from the heart blood in only 1 of 31 cultures. Streptococci were grown from 6 of the 20 spleens that were cultured. The liver was cultured in 12 instances and no organisms were recovered.

These experiments demonstrate that a small number of the streptococci that are injected into the skin enter the blood stream. If it is assumed that they have found their way into the blood by way of the lymphatics, rather than through capillaries at the site of injection, they have not been retained by the superficial or deep lymph nodes. Since a small part of the streptococci that reach the inguinal lymph nodes pass through them to the iliac nodes, it can be assumed that an even smaller number pass from the iliac nodes to blood.

#### Experiments on Sensitized Rabbits

Chinchilla rabbits ranging in weight from 1,600 to 2,200 gm. were sensitized by intracutaneous injections of 0.1 cc. of an 18 hour culture of hemolytic streptococcus given each week during 5 weeks, or later by 0.1 cc. of 1:10 dilution in broth. Skin hypersensitiveness was determined by the extent of erythema and edema, which often surrounded a central firmer area at the immediate site of injection.

homologous streptococcic filtrate. In sensitized rabbits a pink edematous wheal appeared in from 2 to 4 hours and reached a maximum in from 24 to 40 hours. In normal rabbits this filtrate caused only slight diffuse erythema of the skin unaccompanied by edema. In sensitized animals the reaction rapidly receded, but occasionally formed an abscess that persisted as a firm nodule for a month or longer.

In each experiment a sensitized and a normal control rabbit of about the same weight received 0.1 cc. of a 1:70 dilution of an 18 hour culture of hemolytic streptococcus in broth. Of the animals that had received intracutaneous injections of hemolytic streptococci only those that showed acquired skin hypersensitiveness were used. In all instances in which animals lived long enough for a reaction to develop (Table II), the injection of 0.1 cc. of a 1:70 dilution of hemolytic streptococci produced a pink erythematous wheal visible within 4 hours, or occasionally somewhat earlier. In the experiments in which rabbits were killed within 4 hours after inoculation, it was not possible to make accurate comparison of the size of the skin lesions in sensitized and control animals.

In normal rabbits this dosage usually produced only a slight diffuse redness that appeared 12 hours after injection, reached a maximum in 24 hours, and disappeared in 3 or 4 days. In a few normal animals no change of the skin was noted.

In 16 sensitized animals that received 0.1 cc. of a 1:70 dilution of hemolytic streptococci, the lesions varied in size from  $11 \times 11$  mm. with slight elevation, to large edematous and injected wheals measuring  $60 \times 60$  and raised 5 mm. In the control rabbits, save in one instance, they were much smaller and varied from no visible change in a few animals to slight redness with ill defined edema in an area not exceeding 20 mm. in diameter. The one exception was in Experiment 23 (Table II) in which there was unexplained progressive infection in the control animal and an inflammatory reaction that measured  $30 \times 30$  mm. and was raised 3 mm. This unusually severe reaction may have been caused by the great multiplication of bacteria that occurred at the site of injection, or the animal may have possessed the "native sensitivity" described by Kahn (14).

Blood cultures were made from the left ventricle of 15 animals in each group. The spleen was cultured in 33 instances and the liver in 24. The entire spleen was ground in a mortar and 5 cc. of broth were added slowly; 1 cc. of the material in melted blood agar was poured into each of 2 Petri dishes so that the number of colonies on each Petri dish represented approximately one-fifth of the organisms in the spleen. 1 gm. of liver was ground with 5 cc. of broth and was plated as above, the number of organisms in each Petri dish being those in approximately 0.2 gm. of liver tissue.

The results of culturing the skin and inguinal lymph nodes in sensitized and normal rabbits are given in Table II. In 24 experiments the average number of streptococci injected was 199,000.

TABLE II

The Number of Streptococci Recovered from the Skin and Inguinal Lymph Nodes of Normal and Sensitized Rabbits after Intracutaneous Injection of the Organism

	0.1 cc. 1:70 dilution		Normal	rabbits	Sensitized rabbits		
Experiment		NTf	No. of organis	ms recovered	No. of organis	ms recovered	
No.	Time interval	No. of organ- isms injected	Skin	Inguinal lymph node	Skin	Inguinal lymph node	
1	15 min.	678,000	855,000	26	455,000	20	
2	32 "	53,000	982,000	0	311,000	22	
3	1 hr.	42,000	38,000	9	112,000	0	
4	1 "	90,000	19,000	26	9,000	4	
5	1 "	170,000	77,000	18	152,000	0	
6	1 "	148,000	302,000	4	474,000	4	
7	1 "	98,000	116,000	0	,		
8	1 "	63,000	2,900	588	ı		
9	1 "	210,000	276,000	274			
10	2 hrs.	28,000	9,200	4	505,000	0	
11	2 "	76,000	572,000	2	1,158,000	0	
12	3 "	2,200	13,000	161	165,000	0	
13	3 "	478,000	3,020,000	12	11,650,000	0	
14	4 "	468,000	13,620,000	4	14,720,000	0	
15	4 "	99,000	74,000	2	873,000	0	
16	5 "		7,010,000	70	16,860,000	1	
17	7 "	156,000	18,800	19	19,200	0	
18	12 "	678,000	22,000	1	88,500	33	
19	12 "	390,000	200	0	60,000	0	
20	12 "	48,000	0	0	39,000	0	
21	18 "	440,000	0	0	3,700	10	
22	18 "	114,000	0	0	13,600	0	
23	24 "	41,700	5,972,000	30	4,298,000	0	
24	24 "	42,000	0	0	2,000	0	
25	24 "	215,000	0	0	0	0	
26	48 "	365,000	0	0	0	0	
27	72 "	365,000	0	0	0	0	

Site of Injection.—Streptococci were grown from the skin in 21 of 24 sensitized rabbits and in 20 of 27 controls. A larger number of organisms was recovered from the skin of the sensitized than from the

Of the 4 normal rabbits in which there were in the skin more organisms than in sensitized animals, 3 were killed within the 1st hour, namely after 15 minutes, 32 minutes, and 1 hour respectively, and the fourth was the exceptional animal (Experiment 23) that was killed after 24 hours with excessive multiplication of organisms. After the 1 hour interval, with only one exception, more organisms were recovered from the skin of sensitized rabbits. During the period from 1 to 5 hours after inoculation streptococci have evidently multiplied at the site of inoculation. Subsequently there was a gradual decrease

TABLE III

Number of Sensitized and Comparable Normal Control Rabbits with Streptococci in

Lymph Nodes, Blood, or Internal Organs

	Normal rabbits			Sensitized rabbits			
Culture taken from	No. of rabbits Showing hemolytic streptococci in culture		No. of rabbits	Showing hemolytic streptococci in culture			
		No.	Per cent		No.	Per cent	
Inguinal lymph node	27	17	62.9*	24	7	29.1†	
Iliac lymph node	5	2	40	5	0	-	
Blood	16	1	6.2	16	0	-	
Spleen	20	6	30	13	0	_	
Liver	12	0	-	12	0	-	

<sup>\*</sup> Streptococci averaging 46.2 per lymph node.

in number until 24 hours after inoculation, when microorganisms had disappeared.

In the skin of normal rabbits there was considerably less multiplication and few streptococci were recovered from the site of injection in experiments done 12 hours after injection. Later they entirely disappeared except in the normal animal in Experiment 23.

None of the sensitized animals was allowed to live more than 72 hours and no organisms were recovered after 24 hours. Cultures were made in one instance 8 days and in another 23 days after injection of sensitized rabbits, and no growth was obtained. Andrewes, Derick, and Swift (15) recovered green streptococci from lesions of sensitized rabbits 6 days after injection.

<sup>†</sup> Streptococci averaging only 3.9 per lymph node.

From these experiments it is evident that after intracutaneous injections of small doses of avirulent hemolytic streptococci into sensitized rabbits, there is a more acute inflammatory reaction, and in most instances larger numbers of organisms are obtained from the injected skin than from normal rabbits similarly injected. The organisms moreover disappear from the skin of the normal rabbits much sooner after injection.

Inguinal Lymph Node.—There are conspicuous differences in the number of bacteria recovered from the inguinal lymph nodes of sensitized and normal rabbits. Streptococci were grown from the inguinal lymph node in only 7 of 24 sensitized animals, whereas they were recovered from 15 of the 24 controls (Table II). Table III shows the results of cultures made from the lymph nodes of sensitized and comparable normal animals. In no instance were organisms recovered from the inguinal lymph node after they had disappeared at the site of injection.

Iliac Lymph Node.—Streptococci were recovered from the iliac lymph node in 6 of 15, or 40 per cent of the normal rabbits listed in Table I and were present only when there was a fairly large number of organisms in the inguinal lymph node. The iliac nodes from sensitized rabbits (Table III) were all negative, whereas in the control rabbits organisms were recovered from 2 of 5 iliac lymph nodes.

Blood and Internal Organs.—Streptococci were obtained from the blood of only 1 of 31 normal animals (Tables I and III). A few colonies of hemolytic streptococci appeared in the plate from the heart's blood of this rabbit killed 15 minutes after inoculation. Blood cultures from sensitized rabbits were all negative.

The spleens of 20 normal animals were cultured and 6 contained streptococci in moderate number. No streptococci were obtained from the spleens of 13 sensitized animals. Cultures were made from the livers of 12 animals of each group and all were negative.

It is evident that normal lymph nodes of rabbits are fairly efficient filters for hemolytic streptococci injected into the skin and few reach the blood and internal organs. Lymph nodes of sensitized animals are apparently impervious to hemolytic streptococci.

#### Histological Changes at the Site of Injection in Normal and Sensitized Animals

A group of 8 rabbits received each week during 13 weeks intracutaneous injections of 0.1 cc. of a 1:10 dilution of living hemolytic streptococci. The injections were stopped for 7 weeks, and after this interval one more injection was given in order to determine the degree of sensitivity. Shortly after this injection these rabbits together with 8 normal rabbits received an intradermal injection of 0.1 cc. of a 1:70 dilution of hemolytic streptococcus (176,000). The site of injection was carefully marked with India ink, which remained visible until the tissue was ready for sectioning. The rabbits were killed in pairs after intervals of 2, 4, 6, 8, 12, 24, and 48 hours. The skin at the site of injection and the inguinal lymph nodes were fixed in Zenker's fluid and stained with Mallory's eosin and methylene blue. Descriptions of the skin lesions in the two groups of animals follow.

#### After 2 Hours

R. 211 Sensitized.—The lesion measures 11 x 11 x 2 mm. There is diffuse infiltration of polymorphonuclear leucocytes, and scant edema. Many chains of streptococci are seen, and about them polymorphonuclear cells are concentrated, but there is very little phagocytosis. A few eosinophile cells are present. There is slight swelling of the endothelial lining of the small vessels.

R. 337 Control.—There is no visible change at the site of injection. There is slight infiltration of polymorphonuclear cells and a few cocci in chains are seen in the section. The reaction is much less conspicuous than in R. 211.

#### After 4 Hours

R. 255 Sensitized.—The skin lesion measures 20 x 22 mm. There is much edema with distension of the subcutaneous arcolar tissue and considerable deposit of fibrin. There are many polymorphonuclear leucocytes and they show more tendency to collect in foci than at the 2 hour period. Streptococci are numerous, and many of them are adherent to the collagen fibrils. The endothelium of the small vessels is swollen and their lumina are entirely filled with polymorphonuclear leucocytes.

R. 348 Control.—The skin shows slight redness that measures  $10 \times 12$  mm. There is very little edema but a diffuse infiltration of polymorphonuclears and many bacteria are seen singly, in pairs, and in chains. There is no tendency to concentration in any one area. The greater number of organisms lie free in the tissue adjacent to polymorphonuclear cells which have ingested few cocci.

#### After 6 Hours

R. 258 Sensitized.—The lesion is edematous and measures 22 x 27 x 3 mm. A well circumscribed zone of densely packed polymorphonuclear cells with a few mononuclear cells surrounds an area of necrotic tissue in which there are large numbers of streptococci. There is scant phagocytosis.

R.~339~Control.—The lesion measures 11 x 11 mm. There is a small area of necrosis in the epidermis. There is no edema and polymorphonuclear cells are in much smaller number than in R.~258. A moderate number of cocci occur singly and in pairs scattered throughout the section.

#### After & Hours

 $R.\ 290\ Sensitized.$ —The skin lesion measures  $20\ x\ 27\ x\ 5$  mm, and is moderately edematous but not injected. In its central part there is dense infiltration with polymorphonuclear leucocytes, and a considerable number of red blood cells. There is definite necrosis and in this necrotic area there is a small collection of streptococci that has not been invaded by the cellular exudate. There is more phagocytosis than in the other sections.

R. 309 Control.—The skin lesion measures 18 x 5 mm. Microscopically there is a moderate edema and a diffuse infiltration of polymorphonuclear leucocytes, in the center of which there is beginning necrosis, less conspicuous, however, than in R. 290. A moderate number of cocci are seen, but there is little if any phagocytosis.

#### After 12 Hours

R. 292 Sensitized.—Edematous swelling measures 25 x 18 x 4 mm. and there is a well circumscribed abscess in its central part measuring 2 x 3 mm. The normal tissue is replaced by a dense, sharply defined collection of polymorphonuclear leucocytes, in the center of which there is necrosis. At the periphery of the abscess there are polymorphonuclear leucocytes and mononuclear phagocytes in fairly large numbers.

R. 344 Control.—The skin lesion measures 14 x 13 mm. Infiltration of polymorphonuclear leucocytes somewhat more diffuse than in R. 292 and a few mononuclear cells are seen. There is very little edema. Some phagocytosis of cocci is found. Many small vessels are filled with cells and fibrin, and the endothelium is often swollen.

#### After 24 Hours

R. 294 Sensitized.—The lesion measures 50 x 22 x 5 mm. There is a large area of necrosis composed chiefly of degenerated leucocytes and disintegrated collagen fibers. There is edema separating the remaining fibrils. A few cocci are seen singly and in pairs and mononuclear phagocytes are numerous. Fibrin in considerable amount is deposited in several places.

R. 345 Control.—The lesion measures 11 x 10 mm. There is a fairly well local-

ized collection of polymorphonuclear leucocytes surrounded by mononuclear cells that have ingested cocci.

#### After 48 Hours

R. 297 Sensitized.—The skin lesion measures 30 x 40 x 5 mm. There is a large localized abscess with extensive necrosis in its central part. There is active phagocytosis of streptococci and much fibrin is present. A few free cocci are seen.

R.~340~Control.—The lesion measures 8 x 8 mm. A small area beneath the epidermis is infiltrated with polymorphonuclear leucocytes, lymphocytes, and mononuclear cells. A few fibroblasts are present.

The reactions in the skin of hypersensitive rabbits began earlier, proceeded more rapidly, and were accompanied by much more edema than those in the normal controls. Necrosis in the center of the inflammatory area was more extensive and there was a more sharply defined collection of leucocytes. Phagocytosis was equally active in the 2 groups but was not conspicuous in either, though it began earlier in the sensitized rabbits. Deposition of fibrin occurred earlier in the sensitized rabbits. It was not possible to make a valid comparison of the number of bacteria in the different sections of skin, but they seemed to be more numerous in the skin of the hypersensitive rabbits than in the corresponding controls.

The intensity of the inflammatory reaction in the lymph nodes was closely parallel to that seen in the skin from the same rabbit. Both polymorphonuclear leucocytes and mononuclear cells appeared in the lymph node of the sensitized rabbits sooner than in the controls. Many macrophages were present in the lymph nodes of all the animals after 24 hours.

#### SUMMARY AND CONCLUSIONS

- 1. A relatively avirulent strain of hemolytic streptococci injected into the skin of normal rabbits seldom diminished and usually increased in number during the first 5 hours after inoculation but after 12 hours save in exceptional instances the organisms diminished rapidly and disappeared.
- 2. Hemolytic streptococci injected into the skin of normal rabbits passed rapidly from the site of injection to the regional lymph nodes from which they were recovered in considerable number within 15 minutes. Passage of bacteria from the skin of the abdomen to the

inguinal lymph node was greatest within 2 hours after injection and gradually decreased so that few organisms were recovered from the lymph nodes after 7 hours.

- 3. Hemolytic streptococci that reached the inguinal lymph node passed in small numbers to the distal or iliac lymph node, but this passage save in exceptional instances of progressive infection was limited to a period within 1 hour after inoculation.
- 4. Avirulent hemolytic streptococci injected into the skin of normal animals penetrated into the blood; they were seldom recovered from the circulating blood but were more frequently found in the spleen.
- 5. When hemolytic streptococci were injected into the skin of sensitized rabbits they multiplied at the site of injection during the first 5 hours after inoculation and at corresponding time intervals a larger number of organisms were recovered from the sensitized than from normal control animals. Streptococci diminished and disappeared earlier from the skin of control than from that of sensitized rabbits.
- 6. Hemolytic streptococci injected into the skin of the flank of sensitized rabbits reached the inguinal lymph node in much smaller numbers than in normal rabbits, and this passage from the site of injection to the lymph node save in an occasional instance of progressive local infection ceased after the 1st hour. No streptococci passed from the inguinal to the iliac lymph nodes of sensitized animals and none were found in the blood or internal organs.
- 7. Histological examination showed that the inflammatory reaction following the injection of hemolytic streptococci into rabbits sensitized to this organism began sooner than in the normal animal; edema was more extensive and both polymorphonuclear leucocytes and large mononuclear cells appeared sooner. The lesion of a sensitized animal was more sharply circumscribed and there was necrosis of tissue, seldom seen in the normal animals.
- 8. In sensitized animals local injury with necrosis favors the multiplication of relatively avirulent streptococci at the site of entry and explains their survival at a time when they have disappeared in the controls.
- 9. In association with greater local injury and a more intense inflammatory reaction in the sensitized animal as compared with the normal streptococci are fixed at their site of entry; they pass to adjacent lymph

nodes in much smaller number and fail to reach the blood and internal organs.

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## THE MATERNAL TRANSMISSION OF VACCINIAL IMMUNITY IN SWINE

## II. THE DURATION OF ACTIVE IMMUNITY IN THE SOW AND OF PASSIVE IMMUNITY IN THE YOUNG

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Observations on the maternal transmission of immunity to a filterable virus, namely vaccinia virus in swine, were reported in a preceding paper.¹ It was found that pigs which had received colostrum from their vaccinated dams were generally refractory to vaccination during the early days of life, whereas their litter mates which had been raised without colostrum regularly responded to vaccination with a typical cutaneous reaction. It was concluded that the porcine placenta is impermeable to any appreciable amount of the protective substance elicited as the result of vaccination and that the colostrum functions as the vehicle for its transport to the young.

The present report is concerned with 3 additional phases of the transmission of vaccinial immunity in swine, (1) the duration of maternal transfer; (2) the duration of immunity acquired by the suckling young, and (3) the effect of early vaccination on the persistence of this immunity.

#### The Duration of Maternal Transfer

Two immune sows which had originally been vaccinated in the late fall of 1930 were followed through 6 successive farrowings with the view of determining how long they would continue to transmit protective substances to their nursing young.

Suckling pigs from each of the 6 litters farrowed by the 2 sows were vaccinated during the 1st or 2nd week of life. The test pigs were chosen at random and were

<sup>&</sup>lt;sup>1</sup> Nelson, J. B., J. Exp. Med., 1932, 56, 835.

believed to be representative of the several litters. The vaccinia virus<sup>2</sup> was rubbed into parallel scratches made in the skin over the inner surface of the flank. The vaccinated pigs were subsequently kept under observation for a period of 10 to 14 days and during this period they continued to nurse their respective dams.

The results of the vaccination tests on the suckling young from the 2 immune sows, together with the size of the litters and the dates of

TABLE I

The Reaction of the Suckling Young from 6 Successive Litters to Vaccination

Litter No.	Sow No.*	Date of farrowing	No. of pigs	No. of suckling pigs vaccinated	No. of reactors
1	1	Apr. 6, 1931	7	3	0
	2	May 1, 1931	9	3	0
2	1	Oct. 1, 1931	6	3	0
	2	Oct. 18, 1931	8	4	0
3	1	Mar. 20, 1932	6	3	0
	2	Apr. 14, 1932	12	.3	0
4	1	Sept. 9, 1932	6	3	0
	1 2	Oct. 10, 1932	12	4	0
5	1	Mar. 8, 1933	4	2	0
	2	Apr. 28, 1933	10	4	0
6	1	Sept. 5, 1933	7	4	0
	2	Oct. 11, 1933	6	3	0

<sup>\*</sup> The sows were vaccinated in the late fall of 1930.

farrowing are presented in Table I. The pigs from each pair of the 6 litters were uniformly refractory to vaccinia, there being no response to the cutaneously introduced virus in any case. In addition to the young which were tested during the 1st or 2nd week of life a total of 13 pigs, from various litters, were vaccinated at a later time. These pigs likewise failed to show a cutaneous reaction. The suckling young of

<sup>&</sup>lt;sup>2</sup> The vaccinia virus employed in vaccination was obtained from the Laboratories of the New York City Department of Health through the courtesy of Dr. W. H. Park.

non-vaccinated sows, which were tested at intervals as a control or the potency of the vaccinia virus, invariably developed vesicles at the site of vaccination.

## The Duration of the Immunity Acquired by the Suckling Young

With a few exceptions the pigs from the 2 vaccinated sows were allowed to nurse until about the 6th week when they were weaned. In order to determine the approximate duration of the immunity acquired by suckling, representatives from most of the litters were removed at different age intervals and vaccinated.

The results of this experiment are summarized in Table II. teen suckling pigs vaccinated 4 to 5 weeks after birth showed no reaction. Three of 13 weaned pigs vaccinated 8 to 9 weeks after

The Duration of Vaccinial Immunity in the Suckling Young of Immune Sows

Thim.	unity in the Si	ichlina ve	
No tost i	1:	uckling Young o	Immune C.
No. of reactors	4-3 Wks.	8-9 wks.	
No. of reactors	13	1	12-13 wks.
	0 /	13	19
hint m	_ 1	10 papular	14 vesicular
birth likewise failed to react. 7			5 papular
nowever, devolute to react. 7	On . C .		Pupuar

birth likewise failed to react. Ten of the individuals in this group, however, developed small papules. These were generally discrete, showed little or no color, and usually began to retrogress on the 2nd or 3rd day after they appeared. There was little or no scab formation. This is referred to as a papular reaction in Table II and is regarded as suggestive of a declining immunity. Five of the 19 pigs vaccinated after 12 to 13 weeks also showed a papular reaction. In the remaining 14 pigs of this group vaccination was attended by vesicle formation. The vesicles were usually somewhat smaller than those in fully susceptible control pigs and were more commonly discrete. It is believed, however, that the reaction is sufficiently typical to be indicative of a return to nearly complete susceptibility.

# The Effect of Early Vaccination on the Persistence of Suckling Immunity

It was considered of interest to determine whether or not the vaccinia virus which was introduced cutaneously in the nursing pigs

during the first few days of life exerted any immunizing effect in the absence of a visible local reaction. Was the transient immunity acquired by suckling converted thereby into one of long duration? To answer this question suckling pigs which had been vaccinated, without take, shortly after birth were revaccinated 12 to 13 weeks later and their response compared with that in litter mates which had also nursed but had not been previously vaccinated.

The results of the tests on 9 pairs of pigs from 6 different litters are presented in Table III. In eight cases the paired pigs showed practically identical reactions. In one instance only, Pair 5, was a

TABLE III

The Response of Vaccinated and Unvaccinated Litter Males to Vaccination during the
12th to 13th Week of Life

No.	Type of reaction			
110,	Vaccinated young	Unvaccinated young		
1	Vesicular	Vesicular		
2	"	"		
3	Papular	Papular		
4	Vesicular	Vesicular		
5	Papular	"		
6	Vesicular	**		
7	"	"		
8	"	u		
9	Papular	Papular		

difference in the degree of susceptibility indicated. The previously vaccinated pig in this case showed a papular reaction while its unvaccinated litter mate responded with the formation of vesicles.

#### DISCUSSION

Since there is no reason to believe that the suckling pigs which were tested were different in any respect from their unvaccinated litter mates, it can be assumed that their immune dams produced and transported in their colostrum, during the 3 year period of observation, a sufficient amount of protective substance to afford immunity in the one case to 37 and the other to 57 young. These figures represent the total number of pigs farrowed by each of the 2 sows, respectively.

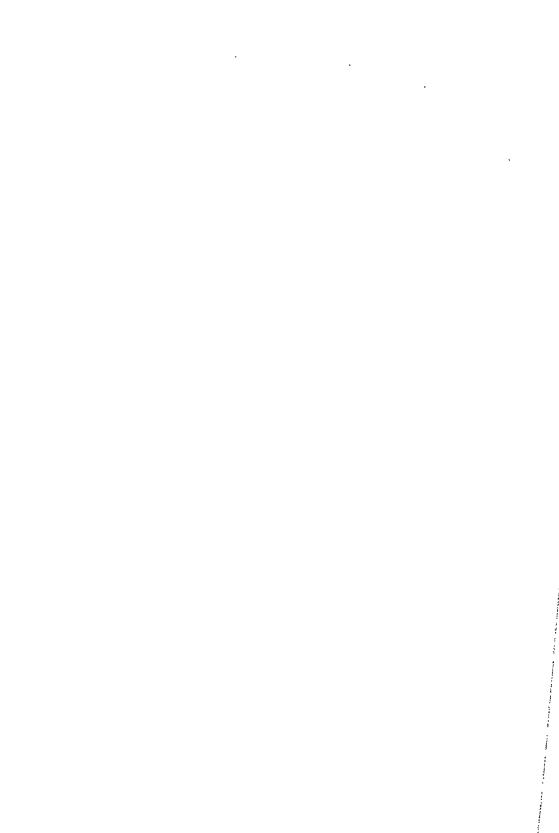
It appears evident that the immune mechanism of the vaccinated sow is able to compensate repeatedly for the amount of protective substance which is lost with each parturition. The actual concentration which is lost in this way must be considerable since protection may be afforded to as many as 12 individuals at the termination of a single pregnancy.

The immunity acquired by the nursing pigs was clearly passive. In most instances complete protection was afforded only during the first 4 or 5 weeks after birth, at least in so far as the concentration of virus employed in vaccination was concerned. The immunity began to decline during the 2nd month and by the end of the 3rd month approximately 75 per cent of the pigs were again susceptible. There was no indication that the decline in protection was any more rapid with the pigs of the 6th litters than with those of the earlier ones. The duration of the suckling immunity was not significantly prolonged by the virus which was cutaneously introduced, without reaction, during the early days of life.

#### SUMMARY

The protective substances produced by vaccinia virus in swine are transmitted repeatedly to the young by way of the colostrum of the sow. In 2 instances suckling immunity was demonstrable in the young of 6 successive farrowings which numbered 37 and 57 individuals, respectively.

The immunity acquired by suckling began to decline during the 2nd month and was practically negligible by the end of the 3rd month. Vaccinia virus introduced cutaneously during the first few days of life in the passively protected pigs exerted little or no immunizing effect.



## A FACTOR FROM NORMAL TISSUES INHIBITING TUMOR GROWTH\*

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(Received for publication, June 21, 1934)

A factor has been demonstrated in certain tumors of the fowl which has a definite inhibiting or neutralizing action on the transmitting agents of these tumors (1). It has proved to be non-specific in its effect, as shown by the fact that it will slow down or actually prevent the growth of a mouse sarcoma (2). It has been suggested that this factor may be related to the control or balancing mechanism of normal cells. The present paper is a report of an investigation designed to test the possible presence of a growth-inhibiting factor in active normal tissues (3).

It might be expected that tissues with the greatest growth energy would require a greater concentration of the hypothetical balancing factor to insure controlled and regulated growth. Therefore, the embryonic tissues have been utilized as the main source of material investigated.

## Material and Method

Several strains of standard transplantable mouse tumors have served as the medium on which the growth-inhibiting factors were tested, the principal ones being the Bashford Adenocarcinoma No. 63, Carcinoma 48, and Sarcoma 180.

Following the method which had given the best yield of the inhibiting substance from the fowl tumors, desiccates of the tissue were employed. The tissues or organs to be studied were removed, minced finely, spread in a thin layer, and placed immediately in a vacuum jar over sulfuric acid. After being evacuated down to about 1 mm. mercury the container was placed in a freezing box and allowed to remain until desiccation was complete. The dry flakes were ground to a fine powder and stored in sealed tubes at ice box temperature until used. The test solutions were prepared by thoroughly extracting 0.1 gm. of the desiccate with 2 cc. of water, by pumping back and forth in a syringe through a coarse needle.

<sup>\*</sup> This investigation was carried out under the Rutherford Donation,

The larger particles were centrifuged out and generally the supernatant fluid was heated at 48°C. for 30 minutes. The Bashford tumor was cut into generous sized grafts and these were nicked in several places to give a greater area of exposed surface. Half of these were placed in the test solution and half in normal salt solution. The time of contact was only that required for loading the grafts into trocars. A certain amount of the extract was carried along with the inoculated material. With No. 48 and the sarcoma, cell suspensions were generally used for inoculation. These were prepared by pressing the tumor through a fine wire mesh, and half of the *Brci* was suspended in the tissue extract and the other half in normal

TABLE I

Effect of Extracts of Mouse Placenta and Embryo Skin on Mouse Tumor Bashford 63

Material inoculated with Bashford tumor graft	No. of experi- ments	No. of mice inoculated	No. of tumors	Average size of tumors	No. negative	Per cent negative
				cm.		
Extract of dry mouse placenta		225	91	$1.02 \times 0.82$	134	59.6
Salt solution (grafts inoculated		211	163	1.43 x 1.10	48	22.7
in same animals as above)	19					
Salt solution (grafts inoculated in other animals)		110	109	$1.73 \times 1.36$	18	16.4
Extract of dry embryo skin		144	64	1.09 x 0.97	80	55.5
Salt solution (grafts inoculated		84	66	1.38 x 1.08	18	21.4
in same animals as above) Salt solution (grafts inoculated in other animals)	10	90	69	1.66 x 1.36	25	27.7
Extract of fresh placenta or	8	79	56		23	29.1
embryo skin Salt solution	°	70	40		30	40.2

salt solution in a ratio of 1:1 between cells and suspending fluid. From 0.05 to 0.1 cc. of these suspensions were injected. In order to secure a more complete check each animal inoculated with the tumor and tissue extract in one groin received the control inoculation of the tumor in salt solution in the other groin. Besides these controls additional animals were frequently inoculated in both groins with the same tumor material in salt solution.

<sup>&</sup>lt;sup>1</sup> It was perhaps not sufficiently emphasized in our earlier papers on the inhibitors in the fowl tumors that the test and the control materials were both inoculated in each fowl, using the intradermal site where accurate measurements may be obtained. By having each test controlled in the same animal the natural variation in degree of susceptibility is eliminated in judging the results.

## The Effect of Homologous Tissue Extracts on the Growth of Transplantable Tumors

The first investigation was carried out with various homologous tissues, but only two strains of tumors were used as indicators.

Experiment.—Fresh tissues were utilized for the earlier experiments and they included fresh mouse placenta, embryo skin, and skinless embryos, with the extracts both heated and unheated. These extracts tested by 222 inoculations of the Bashford 63 failed to show any detectable effect on either the number of takes or the growth rates of the resulting tumors. Likewise, extracts of desiccated skinless embryos and mouse blood, tested by 102 inoculations of the same tumor, showed no influence on the resulting tumors as compared with the controls.

The major experiments were directed towards testing extracts of desiccated mouse placenta and embryo skin, as preliminary tests had indicated an effect on tumors by extracts of these two tissues.

The methods used were those described above. The length of contact between the tumor graft and the solutions was at the maximum 20 minutes. As the heating of the extract at 48°C. for 30 minutes had no material effect on the result all of the experimental results are grouped together. In the majority of instances the control material of tumor immersed in salt solution for a corresponding time was inoculated into the same animal, as well as in other animals. The results, including the average size of the tumor, are given in Table I.

The outcome of a typical experiment is shown in Text-fig. 1.

A similar test was carried out on the effect of the embryo skin and placenta extracts on Mouse Sarcoma 180. The results of these are given in Table II.

The extracts of desiccated mouse placenta and mouse embryo skin give evidence of inhibiting the growth of the Bashford Mouse Tumor 63. The test involved 369 inoculations controlled by 495 inoculations of the same tumor and the effect is shown not only by the material reduction in the number of takes of the treated grafts but by the slow rate of growth of such tumors as were not completely suppressed. In fact there were very few instances in which the tumor arising from the treated graft was not definitely smaller than that from the control graft inoculated into the same animal. There was an absolute failure of extracts prepared from fresh placenta or embryo skin to produce any effect on the growth of this tumor. The Mouse Sarcoma 180 which was definitely retarded by the inhibitor from chicken tumors (2) was unaffected by extracts either from fresh or desiccated mouse tissues.

	Bashford +	grafts +	Bashfor	ed grafts	Additional controls
r	nouse placenta extract	salt solution	mouse embryo skin extract	salt solution	Bashford grafts salt solution
	Right groin	Left groin	Right groin	Left groin	Both groins
1	. <del>-</del>	-	_	-	-
2	<del>-</del>	-	_	_	_
3	_	_	-	-	-
4	<del>-</del>	•	_	_	_
5	<del>-</del>			_	•
6	_		_	•	•
7	_	•	_		•
8			_		•
9	- Marie		_	•	•
10		<b>.</b>	_		
11	_		_		
12	_		_	Ŏ	
13	-		•		
14	•	•	•		
15	•		•		
16	•		•		
17	•		•		
18			•	4	
19	•	•			•
20	•			•	
	65 per cent negative	15 per cent negative	60 per cent negative	25 per cent negative	20 per cent negative

Text-Fig. 1. Effect of homologous tissue extracts on mouse tumor.

## Attempts to Induce General Resistance

While there is nothing in the above described experiments to suggest that the results are in any way connected with the well known induced resistance to transplantable tumors, it seemed advisable to test this point. The method by which this resistant state is brought about is

TABLE II

Effect of Homologous Tissue Extracts on Mouse Sarcoma 180

Material inoculated with Mouse Tumor 180	No. of experi- ments	No. of mice inocu- lated	No. of tumors	Average size of tumors	No. negative	Per cent negative
				cm.		
Extract of dry mouse placenta	10	86	85	2.05 x 1.75	1	1.2
Salt solution	10	69	69	$2.20 \times 1.90$	0	0.0
Extract of dry embryo skin	5	49	49	1.70 x 1.30	0	0.0
Salt solution		50	50	1.70 x 1.20	0	0.0
Extract of fresh placenta	2	12	12	1.80 x 1.55	0	0.0
Salt solution	2	12	12	2.10 x 1.70	0	0.0
Extract of dry skinless embryo	7	59	59	2.35 x 1.85	0	0.0
Salt solution	′	47	47	$2.55 \times 2.15$	1	2.0
Extract of fresh skinless embryo		12	12	2.20 x 1.00	0	0.0
Salt solution	2	12	12	2.10 x 1.70	1	0.0

TABLE III

Effect of Previous Injection of Tissue Extracts on the Growth of Tumors

	No. of experi- ments	No. of mice inocu- lated	No. negative	Per cent negative
0.2 cc. placenta or embryo skin extract in- jected 7 days prior to inoculation of Bash- ford tumor	4	44	14	31.8
Bashford tumor alone (control)	7	34	8	23.5
0.2 cc. placenta of embryo skin extract in- jected 7 days prior to inoculation of Sar- coma 180	2	12	1	8.3
Sarcoma 180 alone (control)		18	0	0.0

the injection of homologous living normal cells into the animal 7 to 10 days prior to the inoculation of the tumor.

Experiment.—Based on four experiments, 44 mice were given subcutaneously 0.2 cc. of either an extract of dry placenta or dry embryo skin, and 7 days later

these mice were inoculated with grafts of the Bashford tumor. At the same time 34 controls were inoculated with the same tumor. A similar experiment was carried out on mice subsequently inoculated with Sarcoma 180. The results of these tests are given in Table III.

There is no evidence from these experiments that extracts of desiccated placenta or embryo skin induce a subsequent general resistance to inoculated tumor.

TABLE IV

Effect of Heterologous Tissue Extracts on the Bashford Tumor

Material inoculated with Bashford 63	No. of experiments	No. of mice inocu- lated	No. of tumors	Average size of tumors	No. negative	Per cent negative
Extract of desiccated rabbit placenta	6	68	23	cm. 1.52 x 1.30	45	66.2
Salt solution	U	68	47	1.97 x 1.40	21	30.9
Extract of desiccated rat	5	59	17	1.10 x 0.90	42	71.1
placenta Salt solution	3	59	43	1.60 x 1.20	16	27.1
Extract of desiccated rat em-	,	40	18	0.90 x 0.70	22	55.0
bryo skin Salt solution	4	40	26	1.40 x 1.00	12	30.0
Extract of fresh rabbit placenta Salt solution	2	17 17	14 13	1.32 x 0.95 1.30 x 1.05	3 4	17.6 23.5

## Inhibiting Action of Heterologous Tissue Extracts

As noted above, the inhibitors from chicken sarcomas are not species limited in their action, as shown by their effect on mouse sarcoma. In the next group of experiments we tested heterologous tissue extracts on transplantable mouse tumors.

Experiment.—In a series of 293 inoculations, controlled by 283 inoculations, the following heterologous tissue extracts showed no very definite effect on either the Bashford Tumor 63 or Carcinoma 48; desiccated and fresh calf thymus, desiccated kidney and spleen of the rat and rabbit, early and term human placenta, and early cow, rabbit, and hog placenta.

The main experiments were tests of rat and rabbit placenta and rat embryo

skin. The methods employed were those described above. The materials were collected during the later stages of pregnancy. The data from tests on Bashford tumor grafts are given in Table IV, and for cell suspensions of Tumor 48 in Table V. The results of a typical experiment are shown in Text-fig. 2.

The inhibiting action of rat placenta and embryo skin and of rabbit placenta is quite definite. As in the case of the homologous tissues the action is manifest not only by the complete suppression of growth in a high percentage, but where the suppression was not complete the growth rate of the resulting tumors was almost invariably distinctly slower than that shown by the control grafts in the same

TABLE V

Effect of Heterologous Tissue Extracts on Mouse Carcinoma 48

Material inoculated with cell suspen- sion of Mouse Tumor 180	No. of experi- ments	No. of mice inocu- lated	No. of tumors	Average size of tumors	No. negative	Per cent regative
Extract of desiccated rabbit	70	275	114	tr. 1.19 x 0.92	161	58.5
placenta Salt solution	28	275	220	1.58 x 1.14	55	20.0
Extract of desiccated rat	2	20	7	1.40 x 1.00	13	65.0
Salt solution	2	20	14	1.50 x 1.10	6	30.0

animals. These points are well shown by Text-fig. 2. The failure of the extracts of fresh tissue are just as evident with heterologous as with homologous tissues. There was no definite effect from extracts of desiccated early cow, hog, or human placentas or from human placentas at term.

## The Effect of Age of Placenta on Inhibiting Action

The absence of any very definite inhibiting action of the extracts of early hog, cow, and human placentas and of human placentas at term opened the question as to whether this was due to the gap between the species or to the age of the placentas. We had some preliminary evidence that early stages of rabbit placenta were not active, while the later stages were active. On the basis of this evidence

of a possible bearing of the age of the placenta on its inhibiting action, a systematic investigation of the point was taken up.

Experiments.—Rabbit placentas were collected at the 12th, 15th, 19th, 22nd, 26th, and 28th day of pregnancy (30 day term) and prepared in the way described above. The extracts of the desiccates were tested on Mouse Tumor 48 in the usual way, with control inoculations of the tumor suspension in salt solution into

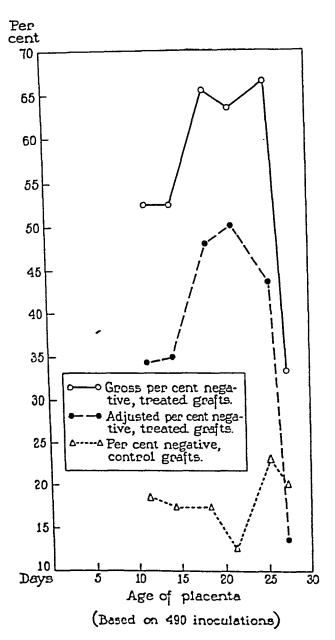
	Mouse Tum + rat placenta extract	or 48 cells + salt solution	Mouse Tum + 12 day rabbit placenta extract	+ salt	Additional controls Mouse Tumor 48 cells salt solution
	Right groin	Left groin	Right groin	Left groin	
1			_		-
2	-	-	_		•
3	-		_	•	•
4	****	•	_	•	
5	-	•	_	<b>©</b>	
6		•	_		
7		•	•		
8	****		€		
9	•		•		
10					
	80 per cent negative	30 per cent negative	60 per cent negative	20 per cent negative	10 per cent negative

Text-Fig. 2. Effect of heterologous placenta extract on mouse carcinoma cells.

the same animals. The results of this test, based on 490 inoculations, are shown in Text-fig. 3. The data for the early stage placentas which were inactive are not included.

In the light of the above described results it would appear that the lack of inhibiting property encountered in the early cow, hog, and human placenta and term human placenta was probably due to the age of the organ rather than to the difference in species.<sup>2</sup> No doubt the

<sup>&</sup>lt;sup>2</sup> Tests on cow placentas between the 7th and 8th month of pregnancy are in progress. The present indications are that appreciable amounts of the inhibiting factor are present.



Text-Fig. 3

curve as represented in Text-fig. 3 might be modified by an increased number of tests, but a sufficient number are available to establish the main points. There seems no immediate explanation in the sudden drop between the 26th and 28th days, 2 days before term.

#### DISCUSSION

This investigation was undertaken on the hypothesis that the balanced state and orderly growth of normal cells is maintained by the interaction of two forces, one which stimulates growth and the other which retards it. Under this hypothesis the break in the balance between the factors, either the loss of the retarding or the accumulation of the stimulating factor, would lead to uncontrolled growth. While the reported results demonstrate the presence of a retarding factor when the extracts of certain active tissues are tested against transplantable cancer—a fact which would seem to support the hypothesis—the question raised is far too complex to justify such a conclusion yet. Nor do we consider that there is sufficient evidence to show that the inhibitor from normal tissues is of the same order or acts in the same manner as the inhibitor isolated from certain fowl However, there are points of probable significance. The inhibitor from the fowl tumors could be demonstrated only in extracts from desiccated tumors, and the normal tissue factor is evident only in desiccates of placenta and embryo skin. With both materials their action is not species limited but appears to be tissue limited, the factor from chicken tumors acting only on sarcomas, and that from placenta and skin only on carcinomas. This specificity of action and the fact that the inhibitors are not demonstrable in extracts of fresh tissues are taken as evidence that they are not simply proteolytic enzymes or some other substance generally injurious to cells.

The explanation of the release of the inhibitor factor by desiccation of the tissues is not evident. It may be that in the tissues it is closely associated with the growth factor which neutralizes its action, and only becomes demonstrable when it is dissociated from the combination through desiccation.

Before the hypothesis suggested can be seriously entertained, the premise that normal cell balance is maintained by the interaction of two forces must be established. While there is a suggestion of some

uch mechanism in the work of Spemann (4) and others the principle still far from being established.

#### SUMMARY

Extracts of desiccated embryo skin and placenta have been found to xert a definite retarding action on the growth of two transplantable arcinomas of mice, but they were without effect on sarcomas. In ests involving some 828 inoculations of the tumor cells and the exracts, complete suppression of growth occurred in from 55 to 71 per cent of instances, as compared with 21 to 27 per cent in the controls, and where growth was not completely suppressed some retardation was found in practically every instance. To judge from findings in rabbits the inhibitor is not demonstrable in the placenta until the beginning of the second third of pregnancy, reaches its maximum by the last third, but disappears about 2 or 3 days before term. Extracts of fresh placenta are without effect, and no very definite inhibition was noted in extracts of a variety of other desiccated or fresh tissues. The conclusions here reported are based on the results of over 3800 inoculations.

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## THE EFFECT OF A GROWTH-RETARDING FACTOR FROM NORMAL TISSUES ON SPONTANEOUS CANCER OF MICE\*

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Extracts of placenta and embryo skin have a definite inhibiting action on the growth of transplantable cancers of mice (1). The absence of any effect of these materials on sarcomas, and the finding that the inhibiting factor isolated from certain fowl tumors acts on sarcomas and not on carcinomas, suggested a degree of tissue specificity for these factors. The attempt to establish a principle applying to cancer in general on evidence derived from the transplantable tumors only leaves something to be desired, for these tumors always represent cells foreign to the host that maintains them. Therefore, we have deemed it necessary to test the above mentioned factor on the spontaneous or natural cancers made up of malignant cells derived from the animal's own tissues (2).

The mammary tumors of mice have been studied because of the number available as by-products of the genetic studies, and because of the extensive data available on the natural history of these neoplasms. There is a surprising uniformity in the figures published by different investigators, using different strains of mice, on such points as the growth of autografts, local recurrence following surgical removal, spontaneous retrogression, and multiple foci of malignant change. In the present communication a report will be made on both the local and general effect upon these natural cancers of inhibiting materials that are known to act on transplanted tumors.

#### Materials

The tumor mice utilized came from three principal sources, and were from several strains. The largest number were from the general breeding room of

<sup>\*</sup> This investigation was carried out under the Rutherford Donation.

The Rockefeller Institute, where the stock of Swiss origin is inbred though not a pure line strain. Figures for the tumor rate, while known to be high, cannot be accurately determined by the available records. The second source was our genetic breeding room, where the mammary tumor strains are descendents of the original Abbe Lathrop stock. The third source, and the second largest, was the Roscoe Jackson Memorial Laboratory, and the animals came from several strains of high tumor incidence. The size of the tumors at the time that the mice were sent into the laboratory was rarely under  $1.0 \times 0.9$  cm. and rarely over  $1.8 \times 1.5$  cm. About 29 per cent of the animals had more than one primary tumor.

The extracts of mouse placenta and embryo skin were prepared in the manner described in earlier publications. The material, collected during the latter part of pregnancy, was minced, spread in a thin layer, and placed in a vacuum jar over sulfuric acid. After evacuation it was kept in the freezing box until desiccation was complete. The finely powdered material was thoroughly extracted with water (0.1 gm. to 1 cc.) and centrifuged. The supernatant fluid was used for the tests.

## Effect of the Extracts on Local Postoperative Recurrence

The early invasion of the skin or deeper tissues by mammary cancer in mice is unusual. Probably owing to the looseness of the tissues, the tumors grow as more or less discrete masses in the subcutaneous tissue and can be easily separated. In spite of the apparent discreteness of the masses and the removal of all immediately adjacent normal tissue, local recurrence is not uncommon. The first tests were on the effect of the inhibiting extract on this manifestation.

Experiment.—The group was composed of 58 tumors removed and the wound bathed with embryo skin extract and 100 tumors removed and the operative field similarly treated with placenta extract. The operation consisted of a radical removal from the etherized animal of the tumor and adjacent tissue, under aseptic precautions. After the wound was sutured, from 0.05 to 0.2 cc. of the tumor extract, depending on the size of the wound, was injected into the area and then it was sealed with collodion to prevent leakage. In the controls collodion was used in the same way. There was rarely any evidence of infection and healing took place promptly. No further treatments were given and the animals were kept under close observation until their death. Diagnosis was made by sections of the original tumors and any nodules found at autopsy were removed and also sectioned. No animals were included as negative for local recurrence unless they survived over 5 weeks. The average length of life after operation was approximately 3 months for the group.

The results are shown in Table I, which for comparison includes our control series in which the tumors were removed without other treatment, two earlier series from this laboratory, and two groups reported by other investigators.

The recurrence rate following operative removal of mouse cancers reported by different investigators with different strains of mice is remarkably constant, the maximum variation between the groups being from 46.2 per cent to 54.0 per cent. The constancy of these figures gives even greater significance to the evidence of suppression

TABLE I

Local Recurrence Following Operation

	No. operated	No. recurred	Percentage recurrence
Controls			
Murray*	48	23	47.9
Haaland†	174	96	54.0
Murphy and Morton‡	. 39	18	46.2
Nakahara§	. 50	26	52.0
Present series	144	69	47.9
Total controls	455	232	50.9
Placenta treated		6	6.0
Embryo skin treated	. 59	15	25.9

<sup>\*</sup> Murray, J. A., 3rd Scient. Rep. Inv. Imp. Cancer Research Fund, London, 1908, 69.

of local recurrence by the two tissue extracts, particularly by placenta extract. While the figures for skin extract show a significant reduction over the expected recurrence rate it is doubtful if this represents the full potency of the material. The larger number of recurrences in this group took place in a lot of animals treated with a single preparation which was probably inactive; but this was not evident until too late to make further tests to establish the point. There seems no doubt that there is a definite reduction in local recurrence due to the treatment of the operative field with the tissue extracts.

<sup>†</sup> Haaland, M., 4th Scient. Rep. Inv. Imp. Cancer Research Fund, London, 1911, 1.

<sup>‡</sup> Murphy, Jas. B., and Morton, J. J., J. Exp. Med., 1915, 22, 800.

<sup>§</sup> Nakahara, W., J. Exp. Med., 1925, 41, 347.

## The Distal Effect of Inhibiting Factors on Tumors

The history of the established spontaneous cancers of mice, particularly the mammary tumors, has been studied by a number of investigators. The most complete data are those collected by Haaland (3) based on 353 tumors and those by Woglom (4) based on 2000 tumors. Haaland found five, or 1.4 per cent which retrogressed more or less completely, while Woglom had only 0.8 per cent which did not show a progressive course. Our figures would be close to Woglom's. We have not seen a well established tumor retrogress among the large number which have been followed in this laboratory. There was a suggestion of an effect on the control graft when the inhibiting extracts were injected into another part of the body, in the test just described. In the next experiments we have considered its possible action on an established primary tumor when injected at a distance.

Experiment.—In this group there were 142 primary tumors, 75 of which were treated by intraperitoneal injection of placenta extract and 67 with embryo skin extract. The injections of 1 cc. were made at weekly intervals, from six to fifteen injections, depending on the response of the tumor. A small piece was removed for diagnosis before the injections were started, in half the animals, and in the other half after the tumor had become stationary, or at autopsy. Otherwise the tumors were not interfered with. As there was no difference in the outcome of the treatments these animals were all grouped together. Weekly measurements were made and complete autopsies were performed at death. No tumors were included in the series unless the animal lived more than 5 weeks, except those dying as the result of growth of the tumors. The general health of the mice seemed unaffected by the treatment.

The results are shown in Table IV.

Among the two groups there were 45 tumors which continued to grow but apparently at a slower rate than in an untreated series. The average progress of 43 of these treated tumors in animals which lived for 4 weeks, compared with 41 of an untreated group is shown in Text-fig. 1. A comparison of this control group with a large number from earlier studies on growth rate shows this to be a fair average.

The growth-inhibiting action of the two tissue extracts is evidenced by the fact that 67 per cent and 69 per cent of the two groups treated showed no further growth of the tumors after the injections had been begun, and may be considered to have lived out their normal span of life, a number of the animals having survived over 6 months and two over a year. The tumors not completely stopped progressed at what appeared to be a slower rate than is usually shown by tumors of this type. The difference in effectiveness between the skin and placenta extracts is not marked here, for the use of an inactive preparation in one of a series of injections would not be so noticeable as in the first two experiments in which a single treatment was given.

TABLE IV

Effect of Embryo Skin and Placenta Extracts on Spontaneous Tumors

	No. of tumors	Con- tinued growth	Sta- tionary or slight retro- gression	Marked retro- gression	Complete absorp- tion	Total showing inhibition
		per cent	per cent	per cens	per cent	per cent
Treated with skin extract	67	32.8	28.4	14.9	23.9	67.2
Treated with placenta extract	75	30.7	21.3	26.7	21.3	69.3

	No.	Original size	2 weeks	4 weeks
Controls	41	1.06 × 0.90 cm.	148 × 1.25 cm.	1.82 × 1.50 cm.
		•		
Treated with skin or placenta extract and growth continued	43	1.13 × 0.93 cm.	1.17 × 0.98 cm.	1.48 × 1.24 cm.

TEXT-Fig. 1. The average growth rate of 43 spontaneous tumors in animals treated with placenta or embryo skin extracts, which continued growth after treatment, compared with the average growth rate of 41 untreated spontaneous tumors.

The number of complete regressions, 32 out of 142 tumors making up the series (a percentage of 22.5) is striking when compared with the fate of tumors in untreated mice in which spontaneous absorption occurs in less than 1 per cent. Tumors showing temporary retrogression followed by subsequent growth are not included among those classed as affected by the treatment. With untreated mice the rule is progressive growth until the death of the animal, but with considerable variation in the growth rate of individual tumors. Cessation of

growth for any great length of time is unusual, and retrogression to any marked degree is rarely seen. This experience is in agreement with Woglom's report, in which among 2000 mice with spontaneous tumors thirteen retrogressed and three fluctuated in growth rate, or remained stationary in size. Thus 99.2 per cent of 2000 untreated spontaneous cancers of mice pursued "their usual progressive course."

## The Occurrence of New Primary Tumors in the Treated Groups

It is well known that mice not uncommonly have multiple primary foci of malignant change in the mammary tissue, the reported figures on this condition yielding an average of about 18 per cent. Haaland (5) collected the first systematic laboratory data and found that after the animal was sent into the laboratory with at least one established primary tumor, some 35 per cent developed additional new tumors before death. It is of interest to analyze the figures for the groups of mice in the above described experiments subjected to treatment with placenta or embryo skin extracts from the point of view of the new tumors developed.

Experiment.—All of the animals in the foregoing experiments were followed closely for their duration of life, and the appearance of new tumors or the finding of them at autopsy was recorded. They may be considered in two groups; one in which the tumors were removed, the operative field treated with one of the extracts, and in which each animal received an autograft which had been immersed in one of the extracts; in the second group the tumors were not removed and the mice received weekly intraperitoneal injections of one of the extracts, the total number varying from six to fifteen. For controls we have two earlier series followed in this laboratory, a recent series the direct control for the present experimental groups, and the figures published by Haaland. The collected data are given in Table V.

Here as with the other data collected from the studies of spontaneous cancer in mice, there is little variation between the figures collected by different investigators and from different strains. The incidence of new tumors in the untreated animals which had previously had one or more tumors, is 37.5 per cent, and the maximum spread between groups is from 32.0 to 43.0 per cent. Among the 210 treated mice the rate was 3.3 per cent, and in the 99 receiving several treatments only

<sup>&</sup>lt;sup>1</sup> Slye believes that pregnancy retards the growth of spontaneous neoplasms in mice (Slye, M., J. Cancer Research, 1920, 5, 25), but as none of the mice in our series were pregnant this point need not be considered.

one new tumor developed, or a rate of about 1 per cent. These figures on the prevention of new foci of malignant change are as definite as those for the other points investigated. The study is being extended to cover the possibility of preventing the origin of a first tumor in mice of families normally showing a high tumor rate. The observa-

TABLE V
Development of New Tumors

	No. of tumor animals	No, which developed new tumors	Percentage which developed new tumors
Controls			}
Haaland*	209	73	35.0
Nakahara*	50	16	32.0
Early Rockefeller Institute series	138	53	38.5
Present series	125	54	43.2
Total controls	522	196	37.5
Tumors removed	{		
One treatment skin extract	46	3	6.3
One treatment placenta extract	65	3	4.6
Tumors not removed		1	l
Several injections skin extract	42	1	2.4
Several injections placenta extract	57	0	0.0
Total treated		7	3.3

<sup>\*</sup> For references see Table I.

tion will include internal tumors as well as those of the mammary gland.

## Histological Study

The tumors composing the above described experimental material represent the usual types of mammary gland neoplasm. Histologically those grafts or unoperated tumors which had been affected by the tissue extracts showed no striking change in their structure. The only prominent feature was the reduction or complete absence of mitotic figures in the tumors after treatment, compared with the biopsy sections. There was a suggestion of more differentiation in the treated tumors and some showed an increase in the connective tissue surrounding and invading the mass, but these differences were

not sufficiently uniform to justify a conclusion as to their importance. No increase in cellular reaction was noted about the grafts, but a systematic investigation of the early changes was not made. A more complete study is in progress.

## DISCUSSION

There are so many gaps in our evidence that it is untimely to discuss the bearing of these results on the hypothesis outlined in the foregoing paper (1), which has served as a basis for the investigation. There is no direct evidence as yet that the factor or substance in embryo skin and placenta extracts is related to the hypothetical control mechanism for normal cells. The lack of any reaction on the part of the host and the fact that the inhibitors are effective when the tumors are treated in vitro before reinoculation suggest that the action is a direct one on the cancer cells rather than a stimulation of the natural resistance mechanism of the body. Stimulation of the latter is the general interpretation accorded to the results obtained with generalized X-ray, dry heat, and fatty acids (6) which seem to represent an entirely different process from the action of the inhibitors. Our present impression is that the inhibitors affect directly the malignancy of the cell. However, any even tentative conclusion must await a more definite understanding of the control mechanism of normal cells.

The findings as concerns the various points investigated, prevention of postoperative recurrence, growth of autografts, the stoppage of further growth of well established cancer, followed in many instances by absorption of the tumor, and the prevention of new foci of malignancy, all indicate a very definite and pronounced effect of the extract of embryo skin and placenta on the growth and development of malignant cells. There were no direct controls for this series in which other tissue extracts were utilized, for such extracts had shown no inhibiting effect on transplanted cancer. It was judged more important to test the two materials which had proved effective with the transplantable tumors on the largest possible number of spontaneous cancers. We have records of unsuccessful attempts to influence the growth of spontaneous tumors by injection of homologous and heterologous serum, red blood cells, and a variety of other protein

materials which would indicate that the present results are not due to some general non-specific reaction.<sup>2</sup>

#### SUMMARY

Extracts of desiccated homologous embryo skin and placenta decrease markedly the rate of postoperative local recurrence after the surgical removal of spontaneous cancer of mice. Autografts after a short period of contact with these extracts either failed to grow, or, in the majority of instances, their subsequent growth was definitely retarded. Intraperitoneal injection of the extracts was followed by cessation of growth of established tumors in more than two-thirds of the animals treated, and among these many of the tumors regressed and over 20 per cent were completely absorbed. Tumor mice treated with either extract rarely developed new malignant foci, though this happened frequently in untreated mice.

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<sup>&</sup>lt;sup>2</sup> This statement is based on an unpublished study of a large series of spontaneous tumors. No doubt other attempts of this sort have been made, and as negative results have not been reported.

## THE SURVIVAL OF VARIETIES OF TYPHUS VIRUS IN MOUSE PASSAGE, WITH PARTICULAR REFERENCE TO THE VIRUS OF BRILL'S DISEASE

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No differences between types of typhus infection were known until 1928, when Mooser (1) recognized the significance of the scrotal swellings observed by Neill (2) and found Rickettsiae in the tunica vaginalis of guinea pigs intraperitoneally inoculated with the Mexican variety of virus. There is no need, for our present purposes, to review the extensive literature dealing with this problem which has accumulated since the publication of Mooser's paper. At the present time, differentiation between the Mexican-American and the classical European varieties by their respective behavior in guinea pigs offers little difficulty to investigators familiar with typhus experimentation. Differences of opinion have, however, arisen within the last few years regarding the relationship between the two types. The point at issue has been whether the observed discrepancies represented temporary modifications of a single type, comparable to the reversible dissociations of bacteria and dependent upon passage through different animal and insect hosts, or whether each variety had become irreversibly fixed in the biological sense. At the Harvard laboratory, at the present time, the view is held that the two varieties are of originally common stock, but that the European type has become stabilized in its present form by continuous passage through man. For this reason it has seemed logical, in agreement with Nicolle and Laigret (3), to speak of the Mexican-American as the murine and of the European as the human virus. This problem has been discussed in greater detail by Zinsser (4) in a paper on Brill's disease which is in press.

<sup>\*</sup> Both authors held Rockefeller Fellowships at the time this work was done.

In the study of this problem, much attention has been given to the behavior of the infectious agents in rodents other than guinea pigs, particularly in rats and in mice. Nicolle (5) was the first to determine the survival of typhus virus, in an inapparent form, in both of these species. That the Mexican-American strain possesses a higher degree of virulence for rats than does the European has been generally recognized since Maxcy (6) showed that definite fever curves resulted from the inoculation of these animals with the first named virus. In a recent study, Laigret and Jadin (7) have reported further differences of considerable importance between the two types in regard to their respective survivals in mouse passage. They carried out mouse passages in series at 9 and 10 day intervals. In one set of experiments they injected brain into peritoneum; in the other, brain into brain. The results of these experiments permitted the conclusions that, by both methods of inoculation, the Mexican virus could be carried from mouse to mouse for at least sixteen passage generations; whereas the human, Tunisian virus was lost after the second passage in the intraperitoneal method, and after the third in brain to brain transmission. These observations are of great interest since, if accurate, they clearly indicate that the observed differences between the two types of virus are not easily reversible dissociations, but are more or less fixed. Incidentally, the method offered an opportunity for further investigation of the virus strains obtained by Zinsser and Castaneda from three cases of Brill's disease.

In 1933, Zinsser and Castaneda (8) reported upon a strain of virus isolated from a typical case of Brill's disease in Boston. In all its attributes in guinea pigs, this virus resembled the European or human variety. Kept alive by guinea pig passage in the Harvard laboratories for 14 months, it has maintained its original character, in spite of repeated experimental efforts to modify it in the direction of the Mexican type. Since the isolation of the first strain, in May, 1933, the same investigators have obtained two further strains from cases of Brill's disease—one in December, 1933; the other in January, 1934. Both of these, like Boston No. 1, have, from the beginning, exhibited the characteristics of the European or human type.

The experiments which are herein reported consisted of a series of mouse passages carried out as follows: The original mouse inoculation

5.15.10	-			
	G. P. 2	Positive	G.P. 3 Negative	G.P. 4 Negative
G.P. 1 1934 M. 2 M. 3	_		M.3	M.3
G.P. 1 1934 Nf. 2	Brains into	Brains into	M. 2 Brains into	M. 2
Bosl G.F Mar. 19, 1934 M. 1 M			M. 1	M.——
	ļ	G.P. 2 Positive	G.P. 3 Negative	G.P.4 Negative
s Strain	<u></u>	M.3	N. 3	M.3
European Strain G.P. 1	M. 2 Brains into	M. 2 Brains into	Mar. 4 Mf. 2 Brains into	Mar. 13
E1 G.1 Feb. 14, 1934	Mf. 1 Feb	M.1.	NI. 1	M. 1
		G.P. 2 Positive	G.P. 3 Positive	G.P. 4 Positive
Strain	M. 3	N. 3	M. 3	M.3
Mexican Strain G.P. 1 1931	M. 2 Brains into	M. 2 Mrains into	Mar. 6 M. 2 Brains into	May 29, 1934G.P., 2
Me G.F Feb. 14, 1934	M. 1 Died			May 29

CHART 1. This chart illustrates the manner in which mouse passage was carried out with three strains of typhus virus, each generation being controlled by guinea pig inoculation. Flerenth generation

Positive

with each strain was carried out intraperitoneally. Three mice were used in each generation, to allow for accidental death and possible irregularities of behavior in individual mice. At the end of 10 days, the mice were killed, the brains pooled, ground in a mortar, and injected intraperitoneally into three, second generation mice and into a guinea pig. This procedure was repeated every 10 days, and the guinea pigs carefully observed by daily temperature and, when appropriate, by *Rickettsia* examination of the tunica vaginalis. When the guinea pig controls of any given generation showed any irregularities of temperature, they were retained and later tested for immunity against the particular strain used for the experiment in which they were controls. The chart shows in graphic form the simple method followed in the experiments.

In this manner, we carried out mouse passages with the Mexican strain originally obtained from Mooser, which is now being used in the Harvard laboratories for vaccine and serum production, and which has been maintained uninterruptedly for about 4 years; with a European strain (Breinl) similarly maintained for about 3 years in this laboratory and, before that, in the National Institute of Health at Washington; and with the three Boston Brill disease strains mentioned above. All of the passage experiments were done in duplicate except in the cases of the Mexican strain and the Boston No. 3.

## EXPERIMENTAL RESULTS

Mexican Murine Strain.—The mouse series was begun on Feb. 14, 1934, by the intraperitoneal injection of tunica material into three mice. Passages were carried out every 10 days from Feb. 14 to May 29, inclusive (the last passage was allowed to go 12 days). The strain is now in its eleventh mouse passage, and the guinea pig controls of every passage, including the last, have reacted typically. It is noticeable, moreover, that the strain has not lost virulence as a result of mouse passage. In the second generation, the incubation time in the control guinea pig was 8 days; by the fourth generation, this had dropped to 7 days; in the fifth and sixth generation, it was 6 days; and in subsequent generations, was 7 days.

While we are continuing to carry on this strain through mice, it seems logical to assume that a virus which can pass through eleven mouse passages without material loss of guinea pig virulence, is not undergoing either attenuation or any other form of modification.

It is apparent, therefore, that our experiments agree with those of Laigret and Jadin in showing that the Mexican virus can be maintained by mouse passage without attenuation and for at least eleven generations—and possibly indefinitely.

European Strain.—Two experiments, in all particulars like the preceding, were carried out with the European strain. The first began on Jan. 15, 1934; the other on Feb. 14. In the first experiment, no guinea pig control was set up for the first generation. In the second experiment, where this was done, the guinea pig reacted positively after an incubation time of 13 days. In both experiments, the guinea pig controls of the third generations were negative, and subsequent tests of these guinea pigs with European virus showed that they had not been immunized by the preceding injection of the mouse brains of the third passage.

This, again, is in agreement with the experiments of Laigret and Jadin, who found that the Tunisian strain of human virus died out after one peritoneal and two cerebral passages.

Brill's Disease Strains.—Experiments like the above were carried out with the three Boston strains, in the case of No. 1 and No. 2 in duplicate.

Boston Strain No. 1.—The second generation guinea pig control was positive in one series after an incubation time of 16 days. In the other experiment, the third generation guinea pig control showed a short lived temperature, but was found to be susceptible on subsequent test. The temperature was, therefore, due to some cause other than typhus infection. In both experiments, the virus had thus died out in the third mouse passage. Passages were nevertheless continued in both series to the fourth and, in one case, to the fifth generation, to insure against possible errors of observation. In neither case did any of the guinea pigs subsequent to the second generation show either typhus reaction or immunity.

Boston Strain No. 2.—The results with this strain were entirely analogous to the ones obtained with Boston No. 1.

Boston Strain No. 3.—Experiments were exactly like the preceding except that, in this case, the reaction in the second generation guinea pig was unusually slight and short lived, so that it could not have been called a typical typhus reaction. This guinea pig was not tested for immunity. Again, however, in the third and fourth generations, the virus died out completely.

#### CONCLUSIONS

The experiments above described have confirmed the observations of Laigret and Jadin that the European human typhus virus cannot be maintained for more than two generations in mice by brain-peritoneum passage; whereas the murine Mexican variety can be carried

on by this method in mice through at least eleven passage generations. The fact that within eleven passages there is no attenuation of the murine virus renders it likely that this agent can continue in mice, in an inapparent form, without material modification.

Brill's disease virus from three different isolations has behaved like the European type, a fact which strengthens the opinion previously expressed from this laboratory that Brill's disease represents an imported European strain of the classical European infection.

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# THE EFFECT OF ANTECEDENT INFECTION AND IMMUNIZATION WITH STREPTOCOCCI UPON THE REACTIVITY OF RABBITS TO HORSE SERUM

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(Received for publication, June 5, 1934)

Tissue reactivity may be altered by infection not only in a specific sense to substances derived from the causative agent, but also non-specifically to unrelated antigenic materials. Specific alterations afford the basis for clinically useful cutaneous reactions in such diseases as tuberculosis, pneumonia, trichophytosis, lymphogranuloma inguinale, and tularemia. On the other hand, non-specific changes in reactivity are involved in fewer clinical demonstrations. Changes in the degree of capillary permeability (1) and in the character of cantharidin blister fluids (2) have been ascribed to alterations in cellular reactivity incident to certain infections.

Non-specific changes in reactivity, although well recognized, have not been studied extensively, and their influence in determining the ultimate response of the body to the introduction of foreign proteins, or of infectious agents seems worthy of additional investigation.

Most observations of this character have been made in experimentally infected animals, and many deal with the effect of tuberculosis upon antigenic responses. Dienes (3) noted that injections of egg white into tuberculous lesions in guinea pigs induced a tuberculin type of skin sensitivity to this substance. Lewis and Loomis (4) observed increased formation of hemolytic amboceptor and agglutinins against B. typhosus following injections of the appropriate antigens into tuberculous guinea pigs. Under similar conditions of infections Selter (5) demonstrated cutaneous hypersensitivity to extracts of colon bacilli. Experiments in tuberculous rabbits dealing only with the rate of production of hemolytic amboceptor have been reported by several observers (4, 6, 7) with highly inconstant or negative results. Certain other chronic infections likewise influence reactivity non-specifically. For example, Lewis and Loomis (8) found accelerated formation of hemolytic amboceptor in guinea pigs infected with streptococci or B. electur. Blumenberg (9) demonstrated tuberculin sensitivity in rabbits when severe chronic

paratyphoid infections were present. Schroeder (10) observed increased hemo

lytic amboceptor production following sheep red blood cell injections into stock rabbits suffering from spontaneous abscesses; but experimental infections with staphylococcus or *B. pyocyancus* did not produce this effect, possibly because the artificial infections were not chronic in character. Wessely (11) in 1911 described an "anaphylactic type" of keratitis that developed after an interval of 2 weeks in a majority of normal rabbits receiving intracorneal injections of horse serum. This reaction was subsequently studied, particularly in luctic rabbits, by a number of investigators with conflicting results (12–16). Among these, vom Hofe and Krantz (16), and Löwenstein (12), found that keratitis developed with greater frequency and intensity in syphilitic rabbits.

It appears, therefore, that certain infections, particularly those chronic in nature, accelerate non-specific responses. This phenomenon, however, has been observed only in the stage of active infection, suggesting that the reaction may be due in large part to transient physiological effects of fever and toxemia rather than to factors exclusively conditioned by infection. That an infection may alter subsequent response, and that this alteration may be of considerable significance is suggested by the fact that non-specific changes of reactivity of decisive influence have been induced experimentally by preliminary injection of antigenic materials.

Semsroth and Koch (17) found that preliminary treatment of guinea pigs with horse serum and of rabbits with streptococcal vaccines or casein markedly altered the reactivity of the endocardium; this was demonstrable upon the subsequent establishment of a staphylococcal infection. Their experience corresponded with that of others to whom they refer (especially Siegmund (18)) in that staphylococcal endocarditis developed regularly only in rabbits receiving preliminary treatments.

Böhmig (19) showed that rabbits injected intracutaneously with small amounts of horse serum reacted subsequently to small intracutaneous inocula of avirulent green streptococci with an inflammation "hyperergic" in character, and succumbed to intravenous inoculations of the same organism in doses which were not fatal for untreated animals.

Daremberg (20) found that the intravenous or subcutaneous injection of 5 cc. of dog serum in guinea pigs infected with human tubercle bacilli occasioned a greatly decreased resistance to later reinfection.

Experiments of Schroeder (10) showed that the production of hemolytic amboceptor, in response to specific antigenic stimulation, was increased by preliminary intravenous injections of pneumococcal autolysates. Similarly, Clark, Zellmer, and Stone (21) found higher agglutination titers for *B. typhosus* after intravenous injections of heat-killed Gram-positive cocci; and Khanolkar (22) encountered a like phenomenon with reference to *B. paralyphosus* after immunizing injections of *B. pyocyancus* vaccine. Clark and his coworkers noted slight increases in resistance following this heterologous immunization. Analogous findings were reported by Otsuki (23) in that resistance to streptococcal infection was increased by preliminary immunization with *B. coli* and *B. proteus*. It is a commonly known fact that guinea pigs treated with diphtheria toxin plus antitoxin are much more highly sensitized to serum than those treated with normal serum or antitoxin only.

#### EXPERIMENTAL

The experiments described in this paper demonstrate altered reactivity in rabbits after the disappearance of mild cutaneous streptococcal infections and following intravenous immunization with streptococci. The critical test was the response to intracutaneous injection of small doses of horse serum. Such a test possesses several advantages. Properly controlled, the results are relatively constant; antigenically, horse serum and streptococci have very little in common; in particular, they provoke contrasting types of sensitivity (24, 25). Although small doses have been employed to demonstrate anaphylactic phenomena in guinea pigs, relatively large quantities have been given heretofore to induce the Arthus phenomenon in rabbits (26). We have found, however, that intracutaneous injections of as little as 0.05 cc. stimulate high degrees of cutaneous sensitivity; areas of edema several centimeters in diameter sometimes follow subsequent injections of 0.01 cc. Small doses (0.1 cc.) were used by Böhmig in sensitization; and recently Kahn (27) noted that quantities as small as 0.5 cc. intracutaneously established cutaneous sensitivity. The desirability of employing the smallest possible doses in testing differences in degree of reactivity is obvious.

Variations in response incident to differences in age, sex, and strain of animals employed are described in another communication (28). The influence of these variables was minimized by employing a constant stock of rabbits consisting of a cross between English, lilacs, and Havanas. Four to six animals were used in each group, and groups to be compared with each other were composed of individuals of approximately the same weight. One lot of serum kept without chemical preservative was used throughout.

The	scheme	of	testing	is	summarized	in	41	£ 11 .	
					CU	111	tne	iollowing	protocol:1

Day	Amount of horse serum intracutaneously	Bleeding to obtain scrum fo
1 3 6 9 12 15 18 21	0. 1 0. 001 0. 001 0. 001 0. 001 0. 001 0. 001 0. 001	Bleeding Bleeding Bleeding Bleeding

A fresh area of skin was selected for each injection. The site of the original injection was observed for 15 days, and those of subsequent injections for 3 days each.

In animals of the character mentioned, the original injection of 0.1 cc. gives rise in 24 hours to a faintly pink papule about 25 mm. in diameter and 1 mm. in height, which fades in 72 hours. In approximately one-half of the animals, however, an edematous lesion reappears at the site of injection about the 9th day and reaches its maximum within 24 hours. This "secondary reaction" is much more intense than the primary reaction and occupies a volume five to ten times as great. It fades completely within a week. The response to the 0.001 cc. injections repeated at 3 day intervals varies. Those made before the 9th day usually provoke no detectable response. Beginning about the 9th day, however, there occur increasingly severe reactions which reach a maximum in 24 hours and fade within In highly sensitive animals, moreover, a delayed reaction is observed following that injection of 0.001 cc. which was made 48 to 72 hours before the development of the secondary reaction above described. Under such circumstances, for instance, the site of the 0.001 cc. injection made on the 6th day remains negative until the 9th day, when a lesion develops concomitantly with the secondary reaction at the site of the original 0.1 cc. injection. As sensitivity to horse serum increases, lesions resulting from the 0.001 cc. injection develop more rapidly and may reach their maximum within 6 hours. Blood serum precipitins are usually demonstrable 24 to 48 hours after cutaneous sensitivity develops.<sup>2</sup> During the course of such injections immature animals gained weight steadily.

<sup>&</sup>lt;sup>1</sup> The intervals indicated here are those suitable for the demonstration of differences in males 2,000 gm. in weight.

<sup>&</sup>lt;sup>2</sup> Precipitin titers were determined by mixing 0.4 cc. of each saline dilution of horse serum with 0.2 cc. of the rabbit serum to be titrated in small precipitin tubes. The following dilutions of horse serum were employed: 1:100, 1:1,000, 1:5,000,

An increased degree of reactivity to horse serum is made evident in several ways: the primary response is greater; the secondary reaction occurs earlier, is found in a higher proportion of animals, and is more intense; reactions to injections of 0.001 cc. occur sooner and are larger; and "delayed reactions" are occasionally observed. The time of appearance of precipitins in the blood serum is advanced. With decreased reactivity these reactions are delayed or absent.

After sensitivity is established according to the foregoing criteria, the injection of 0.1 or 0.01 cc. of horse serum produces areas of redness and edema sometimes covering one entire side of the trunk and occasionally accompanied by hemorrhage and necrosis.

Altered reactivity was induced by inoculating rabbits in various ways with living cultures of an indifferent streptococcus, which is relatively avirulent for these animals, and also by intravenous immunization with hemolytic streptococci. The various forms of altered response with reference to the infecting microorganism have been described elsewhere (29).

Experiment 1.—In Group B, consisting of five females and one male, a state of bacterial hypersensitivity was induced within a period of 22 days by three daily intracutaneous inoculations in 0.1 cc. volume of from  $10^{-2}$  to  $10^{-3}$  cc. of a culture (Strain Q 155) of indifferent streptococcus diluted in Ringer's solution. At the end of this interval, inocula of  $10^{-5}$  cc., which provoked no lesions at the beginning of the experiment, gave rise to distinct papules, and reactions to larger doses were greatly increased. The blood sera agglutinated the infecting organism in dilution of from 1:1,280 to 1:5,120. The animals had, in effect, been subjected to mild cutaneous streptococcal infections for 3 weeks. They gained weight steadily and at the time of the first horse serum injections<sup>3</sup> showed no evidences of persisting inflammation except small cicatrizing papules in two instances. Group A, comprising six females, received no preliminary treatment. The rabbits all weighed from 1,000 to 1,300 gm, each.

The responses to a test of reactivity to horse serum in these two groups are demonstrated in Chart 1. In the controls (Group A) there were no secondary reactions, precipitins appeared in low titer, and in only half of the animals by the 12th day. The 0.001 cc. test doses first provoked small skin lesions on the 12th day.

<sup>1:10,000, 1:50,000, 1:100,000.</sup> The tubes were examined after remaining 2 hours at 37°C, in a water bath, and again after 6 to 8 hours in the refrigerator. The slightest definite precipitate not present in a saline control tube was designated as +; heaviest precipitate (rarely encountered) ++++.

<sup>&</sup>lt;sup>2</sup> Bacterial inoculations were made into the skin over the hind legs and areas over the trunk were used for the horse serum injections.

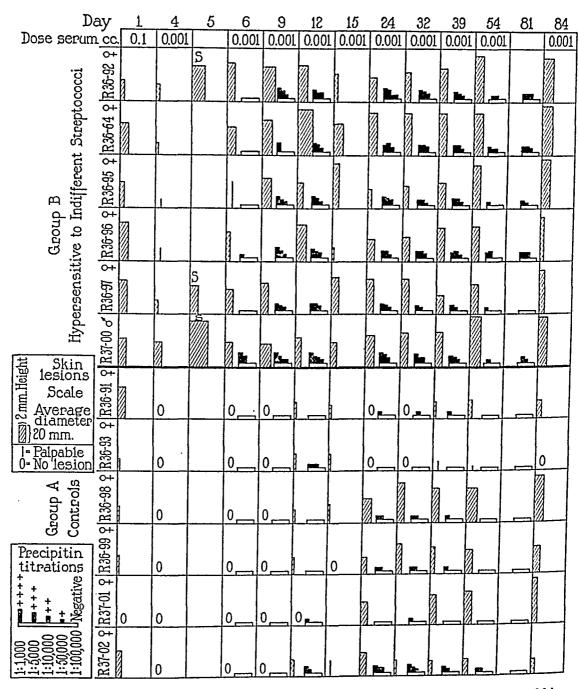


CHART 1. Cutaneous lesions and precipitin titers in control and hypersensitive rabbits following the intracutaneous injection of horse serum. S = secondary reaction.

In contrast, Group B, previously sensitized intracutaneously with streptococci, showed larger original lesions; half of the animals developed secondary reactions on the 4th day; precipitins appeared first on the 6th day and were present in good titer in all by the 9th day. Reactivity to the 0.001 cc. test doses, apparent on the 4th day, became quite marked.

The animals were observed for 3 months; and the following observations concerning the development of skin sensitivity and occurrence of precipitins in the blood serum appear noteworthy.

- 1. Skin hypersensitivity developed before serum precipitins were demonstrable, especially in the sensitive group (B).
- 2. As precipitin titers diminished skin sensitivity often persisted or increased in intensity.
- 3. In individual animals there was no correlation between the degree of skin sensitivity and the height of the precipitin titer.

Experiment 2.—Group B received intravenous inocula of Streptococcus Q 155 in the same doses and intervals as those given intracutaneously in the first experiment (total 0.32433 cc. of culture). Meanwhile, the animals steadily gained weight. Their reactivity to horse serum was tested 5 days after the last intravenous inoculation. Cutaneous reactivity to the streptococci and agglutinin titers were not determined; but it was previously demonstrated (30) that in rabbits so inoculated the agglutinin titers are slightly higher than in those receiving comparable small doses intracutaneously, and that although skin reactivity to inoculation with homologous streptococci is very slightly increased, the lesions are of the type encountered in "immunized" animals.

Group C received large immunizing intravenous injections of living cocci. Rabbits so treated develop serum agglutinins in high titer, and upon intracutaneous inoculation with the homologous microorganism show small indurated lesions (29). In this group some animals lost slightly in weight during immunization, but thereafter gained. There was no obvious focal infection, no arthritis, and at autopsy no scars or collections of pus were found; hence it is improbable that any active inflammation was present at the time reactivity to horse serum was tested, viz. 5 days after the last intravenous inoculation. At this time a previously untreated control group (A) was introduced. Reactivity to horse serum was tested in the manner used in Experiment 1 with the exception that on the 1st day in addition to the sensitizing dose of 0.1 cc. a test dose of 0.001 cc. was also given. The rabbits weighed 1,500 to 2,000 gm. each and the groups were balanced with regard to weight and sex. The results are indicated in Chart 2.

Control Group A.—There was no reaction to the 0.001 cc. injection on the 1st day. Three of the six rabbits developed secondary reactions, all exhibited a degree of skin sensitivity to the small test doses on the 10th day, and the sera of three of the six showed distinct precipitins on the 16th day.

Group B.—One rabbit reacted slightly to 0.001 cc. of horse serum on the 1st day; three of the five developed secondary reactions, one showed a delayed reaction

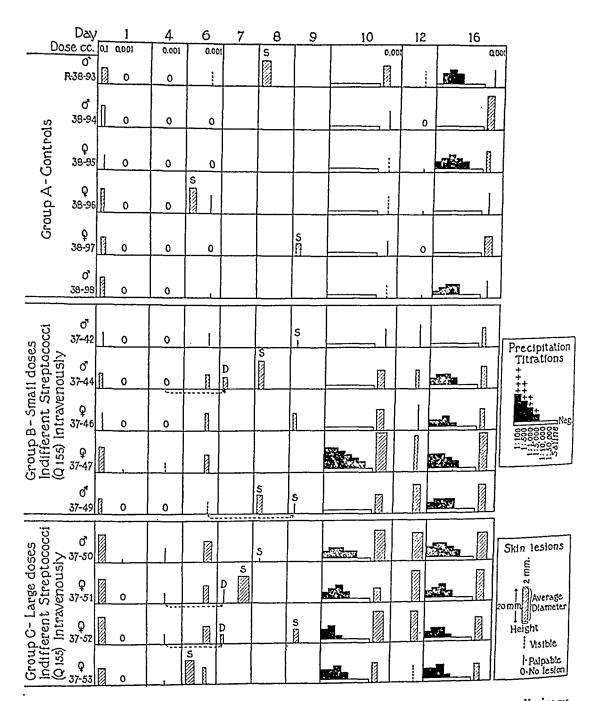


CHART 2. Cutaneous lesions and precipitin titers in rabbits (A) receiving no preliminary treatment, (B) following small doses of indifferent streptococci intravenously, and (C) after large doses of indifferent streptococci intravenously. S = secondary reaction. D = delayed reaction.

to the 0.001 cc. injection on the 4th day, and all gave some inflammatory response to the small test doses on the 6th day. In four of five, precipitins were present on the 16th day, and in one animal precipitins were present in high titer on the 10th day. This group, therefore, showed a very irregular but definite increase in reactivity, although by no means of the grade exhibited in the first experiment by rabbits previously sensitized with comparable doses of the same streptococci intracutaneously.

Group C.—Relatively large lesions resulted from the injections of 0.1 cc. on the 1st day and one animal also responded to 0.001 cc. on this day. Three of the four rabbits developed secondary reactions; all responded to the small test doses on the 4th day (two had delayed reactions to this injection), and all showed distinct precipitins by the 10th day. The increase in reactivity in this group approached but did not equal that shown by the individuals of Group B in Experiment 1 which were subjected to mild cutaneous streptococcal infections for a similar period of time, but which received by comparison only minute doses of the bacteria. This experiment demonstrates the same general phenomena observed in Experiment 1. In addition, delayed reactions to the small test doses were noted frequently, and in some animals showing increased reactivity there was response even to the injection of a 0.001 cc. of horse serum on the 1st day.

Experiment 3.—Here the first two experiments were in principle repeated. One group of rabbits (A) received no preliminary treatment. Group B was sensitized in a manner similar to that used in the first experiment and with cultures of the same strain Each animal received daily intracutaneous inoculations of from 0.01 to 0.05 cc. for a period of 21 days. The total quantity of culture injected was 0.44011 cc. A third group (C) was given large intravenous immunizing doses of hemolytic streptococci (Strain London M A) which was virulent for rabbits. The first injections were of heat-killed vaccine; later, living cultures were used. Eight weekly series of injections were administered and the individual doses were increased from 1 cc. to 3 cc. during the interval. Group B was tested with horse serum 8 days after the last intracutaneous bacterial inoculation and Group C 2 weeks after the final intravenous immunization. At this time the animals weighed from 1,500 to 2,000 gm.; all were gaining weight and there was no evidence of persisting inflammation in any of them. At autopsy no scars or collections of pus were found to indicate that a chronic infection had been induced.

The results are briefly presented in Table I.. The sensitive and immune animals both showed somewhat larger lesions than the controls at the site of the original 0.1 cc. injection. On the 1st day none of the controls reacted to 0.001 cc., while all of the intracutaneously sensitized rabbits and several of the intravenously immunized group showed definite lesions. This order of relative degree of reactivity of the groups, sensitive, immune, control, was also evident in the occurrence of secondary reactions, in the appearance of delayed reactions to 0.001 cc. test doses, in the development of cutaneous sensitivity to repeated test doses, and in the precipitin titers on the 9th day.

The Responses of Control, Hypersensitive, and Immune Rabbits to Small Doses of Horse Serum Intraculaneously TABLE I

Day 1  Second 1	Cutaneous lesions	פתר			
Dose Dose 0.001 cc.  19 x 1+ 0 25 x 1 0 17 x p. 0 26 x 1.5 - 0 20 x 1+ 0 22 x 1- 0 22 x 1- 0 24 x 1.5 14 x p. 25 x 1 24 x 1.5 15 x p. 26 x 1 27 x 1.5 15 x p. 27 x 1.5 15 x p. 27 x 1.5 15 x p. 27 x 1.5 15 x p. 27 x 1.5 16 x p. 27 x 1.5 16 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p. 27 x 1.5 10 x p.		9			Precipitin reaction
Dose 0.001 cc.  19 x 1+ 0 25 x 1 17 x p. 0 26 x 1.5 - 0 20 x 1+ 0 22 x 1- 0 22 x 1- 0 24 x 1.5 14 x p. 24 x 1.5 15 x p. 24 x 1.5 15 x p. 27 x 1.5 15 x p. 27 x 1.5 15 x p. 21 x 1+ 8 x p. 16 x 3 16 x y. 25 x 1.5 13 x p.		Day 4 - Dose 0.001 cc.	Day 6*	Day 9	Day 9
19 x 1+ 0 25 x 1 0 17 x p. 0 26 x 1.5- 0 20 x 1+ 0 22 x 1- 0 24 x 1.5 14 x p. 26 x 1 24 x 1.5 14 x p. 26 x 1 27 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1+ 8 x p. 16 x y. 25 x 1.5 13 x p.	Primary reaction	Delayed reaction on Day 6	Dose 0.001 cc.	Dose 0.001 cc.	1:1,000 dilution
25 x 1 17 x p. 26 x 1.5 - 0 20 x 1 + 0 20 x 1 + 0 22 x 1 - 0 24 x 1.5 14 x p. 26 x 1 24 x 1.5 15 x p. 27 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1 + 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p.	0	0	15 x v.	24 × 14	1
17 x p. 0 26 x 1.5 - 0 20 x 1 + 0 22 x 1 - 0 24 x 1.5 14 x p. 26 x 1 24 x 1.5 15 x p. 26 x 1 24 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1 + 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p.	0	0	0	21 x 0 5	ı
26 x 1.5 - 0 20 x 1 + 0 22 x 1 - 0 24 x 1.5 14 x p. 26 x 1 15 x 1 24 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1 + 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p.	0	0		15 4 4	1
20 x 1+ 0 22 x 1- 0 24 x 1.5 14 x p. 26 x 1 15 x 1 24 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1+ 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p.	0	· c	22 x n	20 * ".	
22 x 1 - 0 24 x 1.5 14 x p. 26 x 1 15 x 1 24 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1 + 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p.	· ·	· c		18 4 0 5 4	! 1
24 x 1.5 14 x p. 26 x 1 15 x 1 15 x 1 15 x 1 15 x 1 15 x 1 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1 + 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p. 23 x 1 + 0	0	0	v. & p.	18 x v.	+
26 x 1 15 x 1 24 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1+ 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p. 23 x 1+ 0	10 % 10	16 * 1	23 🕶 7	70	-
24 x 1.5 15 x p. 27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1+ 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p. 23 x 1+ 0			26 × 1 ×	27 - 2	H - I
27 x 1.5 v. & p. 30 x 2 10 x p. 21 x 1+ 8 x p. 16 x 3 16 x v. 25 x 1.5 13 x p. 23 x 1+ 0	; >	o c	26 × 7	24 X 3	 
30 x 2 21 x 1+ 21 x 1+ 16 x 3 16 x v. 25 x 1.5 23 x 1+ 0	 	15 4 3	1 -	4 6	<del> -</del>
21x1+ 8xp. 16x3 16xv. 25x1.5 13xp. 23x1+ 0	10 × P	17 × 1.	3 6	,	+ ]
16 x 3 16 x v. 25 x 1.5 13 x p. 23 x 1+ 0	14 x p.	19 x 1.5	30 x 2+	20 x p.	+ + + + + +
25 x 1.5 13 x p. 23 x 1+ 0					- -
23 x 1.5 15 x p. 23 x 1+ 0	8 x p.	0	10 x p.	18 x 1+	++
0 +1x57	0	10 x p.	17 x 1	37 x 2	<del> </del>
	v. & p.	1	15 x 1—	56 x 1.5+	l <del>- </del>
0 7 7 7 7	0	13 x p.	18 x 2-	54 x 2	- <del>- </del>
$1-24 = 31 \times 3.5 = 17 \times 1+ = 0$	0	0	23 x 1.5	46 x 5	- <del> </del>

Doses of horse serum intracutaneously (0.1 cc. and 0.001 cc. in 0.1 cc. volume diluted in physiological saline) as indicated. Size of cutaneous lesions: average diameter x estimated height in millimeters.

 $v_{\star} = v_{sib}$ le only.  $p_{\star} = j_{ust}$  palpable.  $v_{\star} \approx p_{\star} = j_{ust}$  visible and palpable. \* Precipitin reactions on 6th day were all negative.

### DISCUSSION

The foregoing experiments indicate that antecedent low grade treptococcal skin infections, as well as intravenous immunization with treptococci, alter the immunological responses of rabbits to horse erum in the direction of accelerated reactivity. They do not, howver, clarify the mechanism responsible for the establishment of such n altered state. Lewis and Loomis (8) have proposed the term "alergic irritability" for a similar condition developing during infection nd have defined it as the "capacity of the animal to react to antigenic ubstances" and "as a general characteristic of the animal on the basis f which it reacts to stimuli of the antigenic class whether they be elpful, injurious or indifferent to bodily health." These authors, as vell as Dienes, Siegmund, and others, have suggested that the stimuation of the reticulo-endothelial system during infection may be reponsible for the phenomena observed. They describe an experiment n which trypan blue injections in rabbits had an effect similar to inection in accelerating the production of hemolytic amboceptor.

The probability of the correctness of such an explanation has increased with the further development of knowledge concerning the unction of the reticulo-endothelial system. The voluminous literature dealing with so called anamnestic reactions is reviewed by Topley (31) and by Weichardt (32). Extensive experiments, particularly those of Walbum (33) and of Weichardt and their collaborators, have indicated that the intravenous injection of metallic salts, colloids, or of protein solutions, may, in certain phases of the immunization process with various microorganisms, increase the titer of circulating antibodies. The significance of such observations indicating that measures affecting the state of reactivity of the reticulo-endothelial system are of decisive influence in altering the immunological response. is discussed in a recent review by Jungeblut (34). The stimulation of this cellular system, as in the demonstration of "anamnestic reactions" results in increased immunological reactivity. Depression has been found to retard the development of skin sensitivity (35) and to prevent fatal tuberculin shock in guinea pigs (36, 37). Numerous studies involving extirpation of the spleen and blockade with various substances indicate that an intact reticulo-endothelial system is essential for normal antibody formation.

Most of the experiments cited have dealt almost exclusively with effects of intravenous treatment or inoculation in altering the functions of the reticulo-endothelial system. The stimulating influence of streptococcal sepsis on this system in the rabbit has been particularly noted (38). That mild local cutaneous infections should have a similar effect might be expected, but our experiments indicate that they exert a considerably more marked influence than that aroused by intravenous stimulation. Tuft (39), recently, in calling attention to the importance of the skin as an immunological organ, suggests that this may be due to the local stimulation of cells of the reticulo-endothelial system which are especially abundant there. The effect of localized inflammation in causing a generalized stimulation of reticulo-endothelial structures has been noted in histologic studies of the liver (40) and of the bone marrow (41), and in physiological experiments (42).

There is abundant evidence that the development of anaphylactic phenomena is distinctly modified by the state of reactivity of the reticulo-endothelial system. For example, Klinge (43) in studying the pathogenesis of the Arthus phenomenon in rabbits sensitized with horse serum, found that the local development of hyperergic inflammation was prevented when the cells of the reticulo-endothelial system, characteristically involved in the reaction, had become impregnated with trypan blue.

It seems justifiable, therefore, to conclude that the accelerated response to intracutaneous injections of horse serum observed in the experiments reported here was conditioned by a state of hyperreactivity of the reticulo-endothelial system which had been induced by the antecedent streptococcal infections and injections. It may be that a similar conditioning of reactivity explains, in part, certain phenomena observed by Jones and Mote (44) in patients suffering from rheumatic fever. These authors noted that such patients became hypersensitive to rabbit serum after receiving repeated intradermal injections of very small amounts of this foreign protein. It seems highly probable that the rheumatic state accelerated and altered the mode of reactivity of these children to the test substance.

An analogy to the phenomena demonstrated in the experiments described is perhaps found in the broadening of the zone of hypersensitivity known from experience to occur in patients. Sensitizations

caused by one substance tend to lose their monovalence and become polyvalent, with a demonstrable sensitivity of the skin, for instance, embracing many substances which may or may not be of clinical significance.

### SUMMARY

- 1. The cutaneous responses of rabbits to small doses of horse serum intracutaneously is described. After an original injection of 0.1 cc. a secondary reaction often occurs about the 9th day, and tests at 3 day intervals with 0.001 cc. quantities indicate that general skin hypersensitivity is established at this time. Circulating precipitins for horse serum appear later. As the degree of sensitivity increases, lesions resulting from test doses reach a maximum development more quickly.
  - 2. The reactivity of rabbits to horse serum is greatly increased by antecedent sensitization of the animals with repeated small intracutaneous inoculation of indifferent streptococci or immunization with large intravenous injections of either indifferent or hemolytic streptococci. Doses of indifferent streptococci precisely comparable to those producing greatly enhanced reactivity when given intracutaneously, increase reactivity to horse serum irregularly and to slight degree when injected intravenously.
    - 3. Increases in reactivity (allergic irritability) are made evident by the occurrence of larger skin lesions at the site of the primary horse serum injections, the earlier development of more distinct secondary reactions in a higher percentage of animals, and by the more rapid appearance of skin sensitivity to the small test doses which may be first evidenced by delayed reactions at these injection sites. Accompanying these evidences of increased reactivity, there is an early appearance of blood serum precipitins in high titer.
      - 4. There was no evidence of residual inflammation in any of the animals at the time reactivity to horse serum was tested. No attempt was made to determine the duration of this altered reactive capacity. It has been encountered in tests begun from 8 to 14 days after the last preliminary inoculation.
      - 5. It is suggested that the increased reaction described is brought about by a persisting alteration in the functional activity of the reticulo-endothelial system.

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# THE INFLUENCE OF SEXUAL MATURITY UPON THE REACTIVITY OF RABBITS TO HORSE SERUM

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Many observations indicate that immunological reactivity is conditioned to some extent by the degree of sexual maturity. In certain tropical and arctic communities where scarlet fever and diphtheria occur very rarely, negativity to both Schick and Dick tests has been found to develop at puberty. Certain dermatomycoses occur exclusively in childhood and heal spontaneously at puberty (1). The development of antibodies against antigen with which contact is not ordinarily established has been found to be similarly conditioned. Conflicting interpretations of such observations are discussed by Manwaring (2), Jungeblut (3), and Herrman (4).

The incidence, severity, and character of the manifestations of rheumatic fever are apparently influenced by factors associated with age and sex. Most observers agree that the disease is chiefly prepubertal in origin, some studies (5, 6) indicating that 98 per cent of first attacks occur before the ages of 12 or 15. These investigations also show that the tendency to relapse is greater in the cases earliest in onset and that in childhood, especially in girls, visceral disease (including carditis) occurs frequently with little or no concomitant arthritis. Chorea develops much more commonly in females but is rarely encountered after puberty except as a complication of pregnancy (7). Wilson, Lingg, and Croxford (6), among others, have stressed the fact that about the 12th year the tendency to rheumatic infection begins to diminish. recent study by von Eickstedt (8) concerning the influence of puberty showed that among 24 girls there were 16 recurrences of the disease in the 4 year period before puberty and none in the following 4 years; among 14 boys the figures were 11 before and 2 afterwards.

The statement is frequently made that the incidence and severity

of rheumatic fever is greater in females than in males of all age groups. Although several general statistical compilations indicate that this is the case, a critical review of the more reliable sources does not substantiate this unqualified conclusion. While most investigations demonstrate that all of the rheumatic manifestations are more frequent and severe in girls than in boys under 12 years of age, those dealing with children under 15 or including all age groups are conflicting in their conclusions regarding the influence of sex factors. relatively greater drop in the incidence of the manifestations of rheumatic fever in females at puberty may be attributed either to a greater influence of the attainment of sexual maturity in that sex or to the fact that while boys and girls are rather equally subjected to environmental factors, different influences are active in the two sexes in adult life; for although women are subjected to the varying influences of the menstrual cycle, men are somewhat more exposed to the stress and strain incident to hard labor and inclement weather.

In the investigations dealing with the influence of age on the formation of natural antibodies or on the response to active immunization (most recently summarized by Freund (9) and Kligler and Olitzki (10)), infants have been compared with adults, and both the intervening age periods and sex factors have, with few exceptions, been neglected. The purpose of the present experiments was to study the influence of the phase of maturation in the two sexes.

Friedberger (11), observing rabbits at intervals from birth to maturity, found that natural anti-sheep erythrocyte hemolysin (amboceptor) which was present at birth, disappeared within 8 days and reappeared thereafter in gradually increasing titer until about the 88th day, with a subsequent slight fall. The time of maximal concentration coincided approximately with that at which sexual maturity is usually established.

Kligler and Olitzki, in titrating antibodies following subcutaneous injections of typhoid vaccine in guinea pigs weighing from 245 to 770 gm., found that the highest titers were attained in animals of 475 to 520 gm. in weight. Agglutinins and complement increased in strength among younger individuals with increasing age: bacteriolysins were first detected in animals weighing 300 gm.; and both agglutinins and bacteriolysins decreased slightly in those over 520 gm. Sexual maturity in

<sup>&</sup>lt;sup>1</sup> Later observations showing differences in immunological reactivity between young and mature rabbits were reported in two abstracts by Baumgartner and by Kahn and McDermott, at the 1934 meeting of the American Association of Immunologists. See J. Immunol., 1934, 26, 327.

guinea pigs, as indicated by the onset of estrus cycles, is attained at the earliest with a weight of 300 gm. and is rarely absent in animals weighing over 500 gm.<sup>2</sup> The maximal increases in antibodies described took place in this interval. In rats ranging from 26 to 114 gm. in weight, similarly immunized, the results were analogous. The highest titers were attained in those weighing between 64 and 83 gm. Sexual maturity in rats (12) as indicated by the onset of an abrupt gain in weight of the ovaries begins when a weight of about 60 gm. has been attained. The relatively greater increases in antibodies in this species began at that point.

Burky (13) observed that rabbits less than 4 months old were insusceptible to the toxic action of a very poisonous broth filtrate of staphylococci, while the majority of rabbits above this age reacted characteristically to intracutaneous injections and succumbed after receiving this toxin intravenously.

The observations mentioned indicate that immunological reactivity is greater in adults than in infants, and that the major increase takes place concomitantly with the phase of sexual maturation. The maximal degree of immunological reactivity, however, seems to be reached just at the point at which physiological sexual maturity is attained, with a subsequent slight decline during continued adult life. It is to be borne in mind that in the experiments quoted no distinction was made between the sexes.

# EXPERIMENTAL

In the experiments described here, rabbits of both sexes and of varying ages are compared in their responses to small doses of horse serum given intracutaneously. The method employed and the character of the reactions studied are described in detail elsewhere (14). Briefly, an original injection of 0.1 cc. is followed at intervals of 3 days by repeated doses of 0.001 cc. The establishment of hypersensitivity to horse serum is indicated by the occurrence of a secondary reaction at the site of the original injection, by the development of lesions following the repeated test doses, and by the appearance of blood serum precipitins. Increased reactivity of the individual is manifest by an acceleration and intensification in the development of the signs of hypersensitivity. The rabbits compared were for the most part of a hybrid English-lilac-Havana stock, bred and raised under identical conditions at The Rockefeller Institute. One group only consisted in adult New Zealand Reds secured from a dealer. Although the age of most of the animals was known, it has been found more convenient to express the degree of development by weight rather than by age.

Experiment 1.—Seventeen male rabbits ranging in weight from 980 to 3,050 gm, were treated with horse serum in the manner outlined. The results are indicated in Table I. In the 13 individuals weighing less than 1,700 gm, no secondary reactions occurred, only small lesions developed in 4 animals following test dozes

Personal communication from Professor C. R. Stockard.

on the 6th and 9th days; and blood serum precipitins were distinctly present in only one instance on the 15th day. In contrast, those weighing 1,700 gm. or more all developed secondary reactions, most of them responded to small test doses given on the 6th day, while all showed quite intense lesions to tests on the 9th day;

TABLE I
Responses of Male Rabbits to Small Doses of Horse Serum Intracutaneously

			Cı	utaneous lesio	Pr	Precipitin reactions			
Rabbit No.	Weight	Secondary		Dose	•		:1,000 di	ntion	
110.		reaction on day	0.001 cc	0.001 cc.	0.001 cc.	] *	. 1,000 Qi	Idrion	
			Day 3	Day 6	Day 9	Day 6	Day 9	Day 15	
	gm.								
R37-87	950	0	0	0	0	_	( <u> </u>	±	
R37-86	980	0	0	0	0		_	+	
R37-85	1,000	0	0	0	20 x v.	-	-	土	
R37-83	1,075	0	0	0	0	-	-	士	
R37-84	1,080	0	0	0	0	-	-	-	
R37-79	1,135	0.	0	0	0	-	±	±	
R37-82	1,170	0	0	0	0	-	-	土	
R37-77	1,170	0	0	0	0	-	- 1	土	
R37-80	1,180	0	0	0	0	-	[	土	
R37-75	1,375	0	0	0	7 x p.	[ - [	- [	土	
R37-03	1,475	0	0	0	0	-	-	土	
R36-79	1,480	0	0	$12 \times p$ .	0	-	<b>–</b> J	士	
R37-94	1,550	0	0	0	21 x 0.5	-	-		
			===				====	1 1	
R36-93	1,700	8	0	15 x v.	24 x 1+	-	- 1	++	
R36-98	1,850	7	0	0	15 x v.	-	+	十土	
R37-33	2,860	9	0	13 x 0.5	22 x 2	-	-	+++	
R37-55	3,050	9	0	20 x 1.5	22 x 1.5+	-		++± 	

Size of cutaneous lesions: average diameter x estimated height in millimeters. v = visible only; p = just palpable.

Animals indicated below the double line weighed 1,700 gm. or more.

and each individual showed precipitins in good titer by the 15th day. An acceleration and an intensification in the development of sensitivity is quite evident in the males weighing more than 1,700 gm.

Experiment 2.—Thirteen female rabbits weighing from 990 to 4,080 gm. were tested in a similar fashion. As indicated in Table II, cutaneous lesions developed

<sup>\*</sup> Doses of horse serum intracutaneously: 0.1 cc. on 1st day, and 0.001 cc. as indicated.

and precipitins appeared irregularly within the group. There was no indication that the degree of reactivity increased with advancing age.

Experiment 3.—In further studies males were compared with females of approximately the same age. The results, including animals used in Experiments 1 and 2, are shown in Table III. In Group A males and females weighing less than 1,700 gm. each are compared. The cutaneous lesions following the test dose on the 9th day were of about equal intensity, and the precipitin titrations on the 15th day were similar. As may be noted also in Experiments 1 and 2, the degree of reactivity was about the same in both sexes before a weight of 1,700 gm. was reached.

TABLE II

Responses of Female Rabbits to Small Doses of Horse Serum Intracutaneously

			Cut	aneous lesio	ons	1	Precipitin	reaction	\$
Rabbit	Weight	Second-		Dos	e .	1:1,000 dilution			
No.		ary reaction	0.001 cc.	0.001 cc.	0.001 cc.		1.1,000	unacon.	
		on day	Day 3	Day 6	Day 9	Day 6	Day 9	Day 12	D2y 15
	£m.								
R36-90	990	0	0	0	$17 \times 0.5$	<b> </b>	_	}	=
R36-78	1,500	0	0	0	0	_	-	} .	-
R36-91	1,500	0	0	0	0	-	-		•
R37-01	1,530	0	0	0	0	-		+	
R36-97	1,550	9?	0	0	$18 \times 0.5 +$	<b>\</b> -	-	]	
R36-93	1,600	0	0	0	0	-	-	+	
	<del></del>								===
R36-99	1,850	0	0	0	0	-	-	\ <del>+.</del>	<b>[</b>
R37-02	1,880	0	0	0	0	-	-	十士	1
R37-98	1,900	0	0	0	0	-	-	i -	
R37-95	1,950	0	0	0	15 x v.	~	-	1	
R37-96	2,000	6	0	22 x p.	20 x v.	-			++
R37-30	3,470	0	0	0	0		-	(	_
R37-31	4,080	0	0	0	0	-		<u> </u>	_

In Group B males and females weighing over 1,700 gm, are compared. The older females developed sensitivity in a manner comparable to the younger individuals of both sexes. In contrast, the males over 1,700 gm, in weight showed secondary reactions, and distinct lesions to small test doses on the 6th and 9th days, while precipitins were uniformly present in good titer by the 15th day. Group C was similar to Group B in respect of maturity, but received an original sensitizing dose of 0.2 cc. instead of 0.1 cc. The same differences in reactivity, however, were manifest: In individuals weighing more than 1,700 gm, sensitivity developed more quickly and more intensely in males.

The results of these experiments indicate that male and female rabbits respond to horse serum in about the same manner before they have attained a weight of 1,700 gm.; that is, to about the age of 13

TABLE III

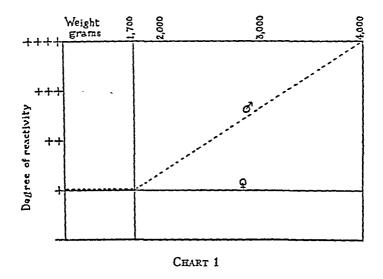
Responses of Male and Female Rabbits of Different Age Groups to Small Doses of

Horse Serum Intracutaneously

						Cutaneous le	sions	Pre	cipitin
	]			ction	<u> </u>	Dos	c	read	ctions
	Rabbit No.	Weight	Sex	y res	0.001 cc.	0.001 cc.	0,001 ec.	1: dil	1,000 ution
				Secondary reaction on day	Day 3	Day 6	Day 9	Day 6 Day 9	Day 15
		gm.	ļ						
	R37-90	990	Female	0	0	0	$17 \times 0.5$	- -	±
Group A	R37-87	1,500	"	0	0	0	0	- -	_
Under 1,700	R37-97	1,550		9	0	0	18 x 0.5+	- -	_
gm. in		,							
weight	R37-86	980	Male	0	0	0	0	- -	+
J	R37-82 1,170 " R37-94 1,550 "		0	0	0	0	- -	士	
			"	0	0	0	21 x 0.5	- -	-
<del></del>								- - -	
	R37-95	1,950		0	0	0	15 x v.	- -	
	R37-96	2,000	"	6	0	22 x p.	20 x v.	- -	++
Group B	R37-30	3,470	"	0	0	0	0 1	- -	_
Over 1,700	R37-31	4,080	"	0	0	0	0	- -	-
gm. in									
weight	R37-93	1,700	Male	8	0	$15 \times v$ .	24 x 1+		++
	R37-98	1,850	"	7	0	0	15 x v.	1,1	+±
,	R37-35	2,860	"	9	0	$20 \times 1.5 -$	$22 \times 1.5 +  $	1 1 '	-十土
	R37-33	3,050	"	9	0	13 x 0.5	22 x 2	- - +	-+± 
	R37-28	4,085	Female	10	0	0	18 x 0.5	_ _	+
Group C*	R37-28	4,190	remaie "	10	0	0	17 x 1	_ _	_
Over 1,700	K31-29	4,190		10		•		1	
gm. in	R37-32	2,830	Male	7	0	20 x 2	32 x 2.5		++
weight	R37-34	3,080	"	8	0	16 x 1	22 x 1.5+  -	<u>- -  -</u>	++ 

<sup>\*</sup> Group C received 0.2 cc. of horse serum on 1st day.

weeks. With advancing age beyond this point only males show an increasing reactivity. The situation may be represented schematically as shown in Chart 1.



with advancing age in the sexes two possible explanations suggest themselves: (1) There are factors peculiar to the male associated with physiological maturation which are responsible for increasing immunological reactivity in that sex. (2) The effect of physiological maturation obtains in both sexes but is not evident in the female because a coincident nullifying influence comes into play. The latter explanation is rendered plausible by the fact that females of the stock employed usually go into heat at the age of 13 weeks, when a weight of 1,700 gm. has been attained, while it is just at this stage of develop-

ment that the divergence between the sexes in respect of reactivity to horse serum is first manifest. In view of this fact, and because the factor of estrus can be subjected to experimental investigation the

following experiment was devised.

In attempting an analysis of the divergence in character of response

Experiment 4.—Twelve adult rabbits (2,300 to 3,000 gm.) were divided into three groups: Group A comprised 4 males which were subjected to simple laparotomy; Group B, 4 females similarly handled; and Group C, 4 females subjected to ovariectomy. 2 weeks after the operations, the animals were all tested with horse serum, and observations were continued for 5 weeks. Because low grade streptococcal infections have been found to increase reactivity to horse serum (13), the wounds were observed closely during life and examined carefully at autopsy to determine whether or not infection had developed. No correlation, however, was

found between the amount of inflammation in the wounds and the degree of reaction to horse serum.

The results of the tests with horse serum are shown in Table IV. One-half the males (Group A) had secondary reactions and 3 of them showed cutaneous reactions to test doses on the 9th day. In one animal precipitins were detected on the 12th day, and they were present

TABLE IV

Responses of Male, Female, and Ovariectomized Female Rabbits to Small Doses of

Horse Scrum Intracutaneously

			Cutan	eous lesions		P	recipit	in read	ctions
	Rabbit	,		Dose			1.1.00	0 dilu	ion
	No.	Second- ary reaction	0.001 cc.	0,001 cc.	0.001 cc.		1.1,00	o unu	
		on day	Day 9	Day 12	Day 15	Day 12	Day 15	Day 21	Day 29
Group A	R39-66	8	20 x 0.5*	22 x 1	24 x 2	+	++	++	+++
Laparotomized	R39-67	9	12 x v.	15 x v.	$18 \times 0.5$			<b> </b>	十土
males	R39-68	0	21 x 0.5	21 x 1	$\overline{18 \times 0.5}$	_		+	++
	R39-70	0	0	18 x v.	18 x p.	-		-	_
Group B	R39-56	10?	v. & p.	22 x 1	32 x 2	_		_	
Laparotomized	R39-59	0	0	0	v. & p.	-	-	-	+
females	R39-61	0	0	26 x p.	$21 \times 0.5$	-	+	++	++
	R39-63	0	0	17 x p.	$\frac{18 \times 0.5}{}$	-	-	+	_
Group C	R39-54	11	10 x p.	15 x 1	20 x p.	-	_	+	土土
Ovariectomized	R39-55	9	13 x p.	$\overline{17 \times 0.5}$	19 x 0.5	- [	-	┼╧│	十士
females	R39-57	8	10 x p.	18 x 0.5	20 x 0.5	-	-	-	+
ļ	R39-60	6	23 x p.	26 x 0.5	25 x 0.5	+	+	+±	++

<sup>\*</sup> Definitely elevated lesions are underlined.

in good titer in 3 by the 29th day. All the castrated females (Group C) showed secondary reactions and slight cutaneous lesions to test doses on the 9th day. Precipitins appeared in about the same concentration as in Group A. The females subjected to laparotomy only (Group B), in contrast to the animals in the other two groups, had no definite secondary reactions, developed no distinct lesions to the test

doses on the 9th day, and showed in only one individual precipitins in concentration comparable to that in the other groups. The findings indicate that adult female rabbits with intact ovaries develop sensitivity to horse serum more slowly and less intensely than do males or castrated females.

At operation the uteri of all the animals in Group B were large and red, indicating that at the start of the experiment all members of this

TABLE V

Responses of Laparotomized and Ovariectomized Female Rabbits to Small Doses of

Horse Serum Intracutaneously

		puu	cent			Cutaneou	s lesions		Precipi-
Rabbit	0	uterus	ht per cight	renc-		1	Dose		tin re- actions
No.	Operation	ht of zina	Uterus weight per of body weight	lary on d	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	1:1,000
		Weight o	Uter	Second tion	Day 5	Day 6	Day 9	Day 12	D2y 12†
		fm.	per cerd						
R38-78	Laparotomy	11.0	0.48	0	0	0	20 x 1	0	_
R38-77	"	11.25	0.42	0	0	0	15 x 0.5	25 x 0.5-	l _
R38-80	Ovariectomy	8.25	0.38	9?	0	0	20 x 1+	35 x 1.5	_
R38-75	Laparotomy	7.5	0.36	9	0	16 x 1-	21 x 1+	24 x v	+
R38-76	"	7.5	0.28	8	$16 \times 0.5$	20 x 1+	33 x 2.5	55 x 2	1++
R38-85	Ovariectomy	5.25	0.20	6	22 x 1.5	18 x 1.5	42 x 3	$40 \times 2.5$	++
R38-86	"	4.0	0.16	6	0	$20 \times 1.5$	47 x 3	53 x 3~	+++
R38-81	"	3.5	0.13	7	12 x v.	17 x 1	23 x 1.5	30 x 2	++

<sup>\*</sup> Weight of uterus and vagina

group were in heat. Autopsies were not performed until  $2\frac{1}{2}$  months later, but at that time the uteri in Group B were large and red in contrast to the atrophic pale uteri in Group C. There was no proof, however, that such a uniform difference existed in the uteri of the two groups several weeks earlier when differences in reactivity to horse serum were being manifest. It may be noted, also, that in general skin lesions were smaller than in unoperated animals, suggesting that a 2 week interval may be an insufficient period for recuperation from the

<sup>100</sup> gm. body weight

<sup>†</sup> Precipitin reactions on 9th day were all negative.

effects of operation in experiments of this character. A fifth experiment was, therefore, devised in which these disturbing factors were reduced to a minimum.

Experiment 5.—Eight adult female rabbits were subjected to laparotomy at which time the ovaries were removed from 4 of them. A period of 1 month was allowed for recovery, and autopsies were performed 13 days after the first injection of horse serum. In other particulars, Experiment 4 was repeated. The results are shown in Table V, ordered according to the degree of estrus present in each animal as evidenced by the relative weights of the uteri at autopsy. Those at the top of the list had relatively large uteri and were definitely in heat; those at the bottom with small uteri were not. It is to be noted that in one instance (R 38-80) the object of the ovariectomy was not accomplished, for although at autopsy no residual ovarian tissue could be identified grossly, the general appearance of the uterus indicated that the animal was in heat. In at least one instance (R 38-76) although the ovaries were intact the uterus was not in the estrus stage.

Probably in consequence of the long recuperative interval after operation in this experiment, cutaneous lesions developed in characteristic fashion. The test with horse serum indicated a distinct difference in degree of reactivity between the animals definitely in heat and those in which atrophy of the uterus had taken place, with a gradual transition between the two extremes. The two individuals (R 38-78 and R 38-77) with very large uteri and intact ovaries had no secondary reactions, showed only slight cutaneous lesions to test doses of 0.001 cc. beginning on the 9th day, and did not develop precipitins by the 12th day. The 3 castrated rabbits with very small uteri (R 38-85, R 38-86, and R 38-81) all had early secondary reactions, showed skin reactivity to test doses of 0.001 cc. beginning on the 5th day and becoming quite intense on the 9th and 12th days, and all developed precipitins in good titer by the 12th day.

### DISCUSSION

These experiments indicate that in male rabbits of the stock employed reactivity to horse serum injected intracutaneously increases with age after 13 weeks, or, in other words, after a weight of approximately 1,700 gm. is attained. In normal females in estrus this increase does not occur with advancing age. Although castrated females showed increasing reactivity coincident with the absence of estrus, the degree of increase was insufficient to account for the divergence exhibited between adult male and female rabbits. It seems probable, therefore, that there may be some additional factor besides estrus to account for the differences in reactivity in the two sexes after attaining maturity.

Friedberger's work demonstrating that in rabbits there is an increase in normal hemolytic amboceptor from the 8th to the 88th days of life with a subsequent slight fall has been mentioned. An inspection of the protocols of his experiments indicates that although the rise in titer was evident in each of the 20 animals studied, the slight fall after the 88th day was confined to the 15 females, while the concentrations of this antibody continued to rise irregularly in the 5 males. In our experiments, the divergence between males and females occurred after the 13th week (91st day). Friedberger's experiments, therefore, appear to indicate that the concentration of normal hemolytic amboceptor is affected by the same factors which influence reactivity to horse serum.

We have found no other record of analogous immunological observations. The experiments of Barron (15) indicate, however, that reactivity to drugs may be conditioned in a comparable manner by the character of sexual maturity. In administering sodium amytal to rats varying between 30 and 400 gm. in weight, he found that the quantity required to produce deep non-fatal anesthesia became greater with increasing weight in males. Females responded similarly until a weight of 60 gm. was reached, but thereafter they required a relatively smaller quantity. The tolerance of males beyond this period, on the other hand, increased sharply. This sex difference appeared at an age when the ovaries begin to increase rapidly in weight.

### SUMMARY

- 1. Normal rabbits in the course of their development, after infancy, vary in their responses to minute doses of horse serum given intracutaneously.
- 2. There is increased reactivity in males as they mature, beginning. in the stock employed in these experiments, at about the age of 13 weeks when a weight of 1,700 gm. has been attained.
- 3. The reactivity of normal females remains at a constant level and the increase demonstrated in males does not take place.
- 4. The divergence in degree of reactivity between males and females, beginning with the establishment of estrus, indicates that factors associated with sexual maturity in females are responsible for their low reactivity.

5. The suppression of estrus by operative removal of the ovaries results in an increase of reactivity which, however, in the experiments reported here is probably not great enough to account entirely for the difference in reactivity between mature males and females.

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# STUDIES ON ORGANOGENESIS

# I. THE ABILITY OF ISOLATED BLOOD CELLS TO FORM ORGANIZED VESSELS IN VITRO

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PLATES 13 TO 15

(Received for publication, June 21, 1934)

Blood cells, when placed for incubation in culture flasks containing a mixture of blood plasma and Tyrode solution, are capable of constructing highly organized, tubular processes that resemble, in some respects, the blood capillaries of the organism. It is the purpose of this communication both to describe these structures and to disclose the conditions under which they are formed.

### Materials and Procedures

The cells are taken from blood that has been drawn from the carotid of adult chickens according to the procedures regularly employed in the preparation of plasma. The blood is collected in plain glass tubes and centrifuged immediately. No anticoagulant is used. After centrifugation, and the subsequent removal of the cell-free plasma yield, the thin layer of leukocytes (buffy coat) that rests above the red cells is coagulated by the addition of a drop or two of embryo tissue juice.\(^1\) A little later, this layer can be removed as a disc and cut into small fragments for the preparation of the cultures. Each fragment consists of a layer of white cells, together with such red cells as remain adherent to them. After a brief period of irrigation with Tyrode solution, the fragments are placed in micro flasks\(^1\) in a medium consisting of 0.2 cc. of adult chick plasma diluted with twice that amount of Tyrode solution. They are then placed for incubation at 37°C.

<sup>&</sup>lt;sup>1</sup> This material is rendered cell-free by freezing for two periods of 15 minutes each at -50°C.

<sup>&</sup>lt;sup>2</sup> This flask (Carrel, A., Compt. rend. Soc. biol., 1930, 105, 826) is 30 mm. in diameter and 5 mm. in depth. It has a straight neck, 5 mm. in diameter. At the union of the neck with the chamber, there is a slight constriction which prevents the medium from running into the neck. The walls are of sufficient thinness (0.18-0.10 mm.) to permit the study of cultures with a 3 mm. oil immersion lens. At the termination of an experiment, the cultures may be fixed and stained in situ.

Early Stages in the Development of the Tubules .- As a rule, the tubules (Figs. 1-4) begin to be formed as soon as the cultures have been prepared. At one or more points on the margin of the implanted fragments (Fig. 4), a few red cells become dislodged and break away. Their places are taken by those from behind. If the proximal impact is great enough, these cells are also pushed out into the medium. This makes for a general streaming. Each cell that is forced out follows in the wake of those that have gone before. They may proceed in single file, or abreast of one another, but always over exactly the same route. This route may take any direction. It may become so branched as to form a system of great complexity (Fig. 1). But as long as the cells in the lead are being pushed forward by those from behind, they will continue to advance through the medium until it coagulates. The moment coagulation occurs, the pathway assumes the nature of a tunnel-like passage. Moreover, this passage will remain open and unobstructed, despite the fact that the cells in transit may be widely separated (Figs. 11 and 12). Its boundaries become the interphase between the fluid plasma within and the coagulated plasma without. By manipulating the flask, the cells that are enclosed can be made to flow in either direction. If coagulation occurs before the proximal outflow has ceased, its lumen may become densely packed with cells. Even after the medium has coagulated, however, the force of the outgoing cells may still be so great that a large spherical expansion is formed at its distal end (Fig. 3). At other times, the channels may be completely ruptured.

It has already been stated that the marginal outflow is initiated by the red cells. Red cells have smooth surfaces and easily shift their position. Without them, the phenomenon does not occur. Leukocytes, on the other hand, adhere to one another and to solid structures. While capable of independent locomotion, they may, as in the present instance, be swept along in a current of red cells. The outflow will not occur, however, if the buffy coat has become too firmly coagulated before the fragments have been prepared and placed in the flasks. Under these conditions, the red cells are held so securely in place by the fibrin meshwork of the explant itself that they are rendered incapa-

ble of movement. The greatest number of tubules are to be obtained from a thick fragment of relatively loose texture and one that contains an abundance of red cells.

It should also be pointed out that the tubule formation occurs independently of, and begins prior to, the general outward migration of leukocytes (Figs. 4 and 5). In fact, the two phenomena depend upon entirely different environmental conditions. It has been found, for example, that when the medium is composed of serum, the cells flow out irregularly from all sides, and no tubules are formed. In the presence of plasma, however, the outflowing cells may take every conceivable form, ranging from broad, fan-like disseminations through short, stalky, bud-like projections to long, slender ones such as those just described. It has also been found that the development of the tubules is not suppressed by substances that enhance cell activity unless they induce, at the same time, the immediate coagulation of the plasma. Thus, embryo tissue juice prevents their formation by producing immediate coagulation (Figs. 6 and 7). Their development is not hindered, however, by the addition of tryptic digests of fibrin. Although these latter substances stimulate the leukocytes to enormous activity, they do not hasten, to any appreciable degree. the coagulation of the fibrin network. It is, of course, possible to delay, to some extent, the onset of coagulation by retaining the cultures for a time at room temperature. This, as a rule, makes for the development of a larger number of tubules and for tubules of greater length than it is possible to obtain by immediate incubation.

Later Stages in the Development of the Tubules.—All that has occurred up to this point may take place within 15 to 20 minutes after the cultures have been prepared. As soon as the surrounding medium has become firmly coagulated, however, no further change occurs either in the length of the tube or in its diameter (Figs. 9 and 10). So far, in fact, the cultures have no definite walls. In the majority of cases, the walls begin to be laid down after from 5 to 8 hours' incubation (Fig. 8). They are formed by the activity of living cells, or cell products, particularly the thrombocytes, that have been deposited along the course of the tubules. Wherever there are gaps between the cells confined in the tubules, these minute bodies may be seen to

spin out fine, hair-like filaments at the interphase between the lumen of the tubule and the surrounding coagulum (Figs. 11 and 12). These filaments are of the nature of fine mesenchymal fibrillae. Sometimes, they become very dense (Fig. 13). They take on a deep blue coloration when stained with Heidenhain's azan mixture, and become black when treated according to the Bielschowsky-Maresch silver impregnation procedures (Figs. 21 and 22).

Out in the surrounding medium, the thrombocytes may form similar strands, but here at random (Figs. 14 and 15). Where they are well isolated from one another, these strands are usually very short and are arranged radially (Fig. 14). The majority of them, however, tend to agglutinate. Very often, the agglutinated masses become joined together by numerous, parallel threads (Fig. 15).

After a few days, the red cells within the capillary-like formations become progressively phagocytized by the macrophages (Figs. 10 and 19). Eventually, these in turn may escape into the surrounding medium, sometimes through definite breaks in the walls, but more often by way of their proximal ends. After some days, the tube may be completely empty. In the meantime, however, any number of macrophages may come to rest on its outer surface (Figs. 16, 21 and 22). In this position, they bear striking resemblance to the much discussed cells of Rouget. Occasionally, their undulating membranes become so fused with one another as to give the impression of an unbroken syncytium. If the cultures were fixed and stained at this moment, the walls of the vessels would appear to be nucleated.

Sometimes the fibrous wall is incompletely formed, the strands being laid down over a single portion of the original passageway. At other times a single cord of fibrous material may extend along one side of it (Fig. 18). Invariably, however, its lumen remains open and filled with fluid. When the wall is complete, it is possible to inject the tubule with a micro pipette. With increasing pressure, the vessel becomes distended. Eventually, of course, it will burst.

### DISCUSSION

The present experiments have shown that it is possible for the cells of the circulating blood, when placed for incubation in a suitable ubstratum, to provide themselves with organized vessels3 of their wn creation. In the beginning, the formation of the organized strucures is a purely physicochemical phenomenon. It is dependent upon he consistency of the medium, the character and thickness of the original fragment and the surface peculiarities of the red cells. As soon as fragments containing a loose arrangement of red cells and eukocytes have been placed in a plasma mixture that does not coaguate immediately, certain of the red cells, as the result of physical pressure exerted upon them by neighboring cells, initiate a general outflow from the central mass into the surrounding medium. This leads to the formation of definite channels, or passageways, through the uncoagulated plasma. Eventually, the medium coagulates. Despite this, however, the plasma contained in the tubular channels remains fluid. Its properties have been altered, in some way, by the advancing blood cells. Then, as soon as the tubules have been formed. the thrombocytes that have been deposited at the interphase between the coagulated plasma without and the fluid medium within, proceed to lay down a fibrinated wall. They build this wall in exactly the same way that they produce at random short strands of fibrillae in the outer medium. Finally, the wall becomes covered by a membranous layer of leukocytes. Here again, the leukocytes manifest a common, well known property. They will cover a cotton fiber in quite the same manner (Fig. 17).

It follows, then, that the phenomenon as a whole is composed of a series of events that are both physicochemical and physiological in their nature. These various events are separate and distinct phenomena. They must, however, occur in a definite sequence. If, for example, the blood cells fail to stream out from the central explant before the medium coagulates, no tubules are formed. The streaming

<sup>&</sup>lt;sup>2</sup> Two years ago, Hueper and Russell (Hueper, W. C., and Russell, M. A., Arch. exp. Zellfersch., 1932, 12, 407) reported the appearance of similar structures in a certain percentage of "leucocyte" cultures, some of which had been prepared in much the same manner as those referred to above. The observations reported by these authors, however, are so completely at variance with those presented here that it does not seem advisable to attempt a comparison of the two accounts. Moreover, no pretence was made by them to determine the conditions under which the phenomenon occurred.

has been found to depend upon the physical features of the explant and the cells that comprise it; the rapidity with which the medium coagulates, on the other hand, depends upon the physicochemical nature of the various substances that go into it and also upon the temperature at which it is placed. Then again, the marginal outflow from the explant into the medium may carry only red cells. While this will insure the formation of the fluid channels, or passageways, through the plasma coagulum, the channels will not become endowed with fibrillar walls unless the red cells are accompanied by leukocytes and thrombocytes. Assuming, however, that the tubules have been formed and that they have developed fibrillar walls, the possibility of their being covered, eventually, by a cellular membrane depends entirely upon the nutritive quality of the medium.

The formation of these structures is looked upon as a definite example of organogenetic development. The phenomenon takes place whenever a certain group of cells is placed in a certain type of medium. Given the proper set of environmental conditions, its occurrence is inevitable. It is true, of course, that the structures formed are only slightly analogous to the blood vessels formed in the organism. At the same time, their manner of formation has been such as to suggest that formative processes occurring in the organism might also depend upon just such an ordered sequence of physicochemical and physiological events.

## SUMMARY AND CONCLUSIONS

- 1. Isolated blood cells, when placed for incubation in a plasma substratum, are capable of constructing highly organized, tubular processes that project from the explanted cell mass into the surrounding medium.
- 2. The tubular structures have fibrillar walls that may be covered, eventually, by a membranous layer of leukocytes. Their lumina contain blood cells suspended in fluid.
- 3. The formation of the tubules is initiated by the red cells. The leukocytes, and more particularly the thrombocytes, are responsible for the construction of the walls.
- 4. The phenomenon occurs only in the presence of plasma, the coagulation of which has been slightly delayed. Once the surrounding

medium has become firmly coagulated, no further change occurs either in the length of the tubules or in their diameter.

- 5. The development of the tubules is not suppressed by substances that enhance cellular activity unless they induce, at the same time, the immediate coagulation of the medium. The addition of embryo tissue juice prevents their formation by producing early coagulation.
- 6. The phenomenon as a whole is dependent upon the physicochemical nature of the medium, the character and thickness of the explanted fragment, and the physical and physiological peculiarities of the cells that comprise it. It is the expression of various physicochemical and physiological events that have occurred in a definite order, or sequence.

The author is greatly indebted to Mrs. Jane Bell Gladding, who has performed a large portion of the experimental work.

### EXPLANATION OF PLATES

With the exception of those reproduced in Figs. 21 and 22, all of the photographs were made from living, unstained preparations. Eastman safety process films were used throughout.

### PLATE 13

- Fig. 1. Culture 5308-4. Tubule formations in a colony of blood cells photographed after 24 hours' incubation in plasma and Tyrode solution. The cells in the background are leukocytes that have migrated out from the same explant. ×31.
- Fig. 2. Culture 5589-3. Portion of a tubule after 24 hours' incubation. The wall, clearly defined where it is present, is still incomplete. Note the miscellaneous character of the enclosed cells. ×198.
- Fig. 3. Culture 4048-8. Tubule from a culture incubated for 30 hours. Note the spherical expansion at the distal end. ×99.
- Fig. 4. Culture 4078-1. Tubule formations after 1 hour's incubation. The structures extend well beyond the zone of migrating leukocytes. ×28.
- Fig. 5. Culture 4078-2. Tubule formations after 8 hours' incubation. By this time, the zone of outward cell migration extends well beyond the tubules.  $\times 20$ .
- Fig. 6. Culture 4092-10. Tubule formations in a colony of blood cells cultivated in plasma and Tyrode solution. As usual, no embryo tissue juice was added. Photographed after 5 hours' incubation. ×17.
- Fig. 7. Culture 4092-3. Colony of blood cells cultivated in plasma, Tyrode solution and embryo tissue juice. In contrast to the culture shown in Fig. 6, no tubules were formed. Photographed after 5 hours' incubation. ×17.

Fig. 8. Culture 4048-7. Portion of a tubule after 6 hours' incubation. In this section of the tubule, a few red cells are to be seen. A spherical expansion at its distal end (top of figure) is not shown.  $\times 164$ .

## PLATE 14

- Fig. 9. Culture 4048-7. The distal portion of a tubule photographed after 30 hours' incubation. The red cells contained in the distal expansion are evenly distributed. ×105.
- Fig. 10. Culture 4048-7. The same as Fig. 9 after 3 days' incubation. By this time, the red cells have become phagocytized by the leukocytes. The several black blotches represent the location of phagocytes that are gorged with red cells. The proximal portions of the tubule are almost completely empty. ×105.
- Fig. 11. Culture 4078-2. Portion of a tubule photographed after 8 hours' incubation. That section of the passageway lying between the two groups of enclosed red cells is open and unobstructed. Although the wall has not yet been formed, a single fibrous strand may be seen extending along the bottom of the tubule. ×365.
- Fig. 12. Culture 4078-2. Portion of another tubule from the same culture after 8 hours' incubation. As in the case shown above, this tubule extends across the entire width of the photograph. The fibrous wall is in process of formation. Only those cells that are clearly in focus are contained within the tubule.  $\times 365$ .
- Fig. 13. Culture 4097-2. Portion of a tubule after 5 days' incubation. The fibrous wall is completely formed and is very dense. ×328.
- Fig. 14. Culture 5016-3. Red cells, leukocytes and thrombocytes from the outer medium of a culture incubated for 24 hours. The thrombocytes have thrown out fine fibrillar strands that radiate in every direction. Just above the center of the figure a macrophage is seen clinging to one of the heavier connecting fibers. ×310.
- Fig. 15. Culture 5016-3. Agglutinated masses of red cells, leukocytes and thrombocytes from another portion of the same culture after 24 hours' incubation. The various groups of cells are connected with one another by numerous fibrillae. ×310.

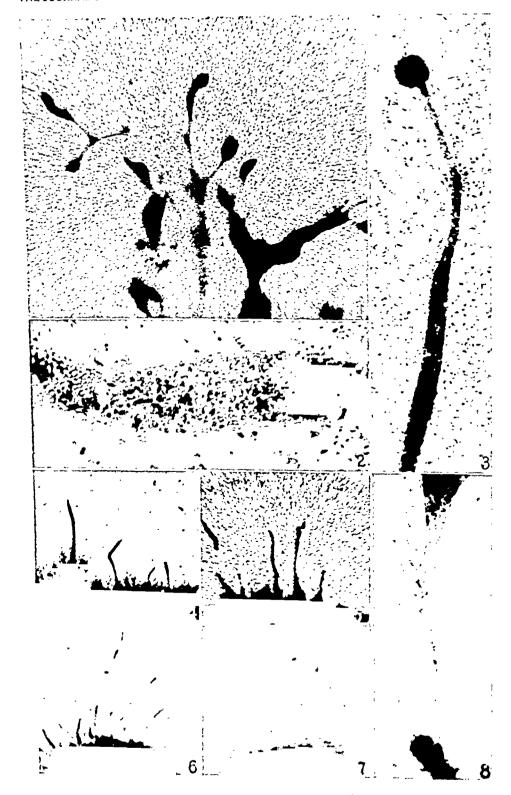
### PLATE 15

- Fig. 16. Culture 4048-8. Portion of a tubule after 5 days' incubation. Its surface is completely covered by leukocytes. Their thin, undulating membranes have become fused; giving the appearance of a syncytium. At the bottom and to the right of the figure, one of the cells lying on the surface of the tubule may be seen in relief. ×207.
  - Fig. 17. Culture 4087-10. Two sections of a single cotton fiber that has served as a supporting structure for free wandering leukocytes; from the outer medium of a culture of blood cells after 6 days' incubation. ×198.
    - Fig. 18. Culture 4048-9. Portion of a tubule whose cellular contents have

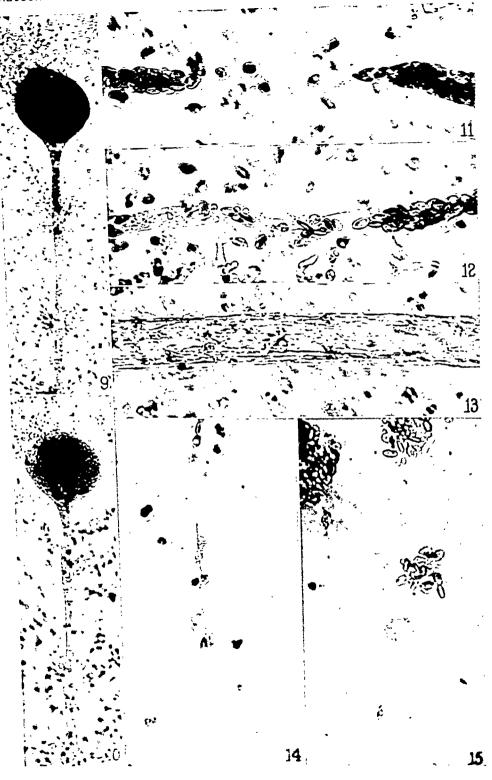
either died or migrated out from its lumen. Only a section of the tubule has been enclosed by a fibrous wall. Although the original channel branched out to the right (at the bottom of the figure), no wall was formed. Photographed after 8 days' incubation. ×185.

- Fig. 19. Culture 4048-7. The distal portion of the same tubule shown in Figs. 9 and 10, after 8 days' incubation. ×185.
- Fig. 20. Culture 4048-7. The distal portion of a tubule that has obtained a well formed, fibrous wall. The extreme tip of this tubule is of very small diameter. Photographed after 9 days' incubation. ×185.
- Fig. 21. Culture 5925-3. Portion of a tubule from a culture fixed in Ringer-formol after 5 days' incubation and stained according to the silver impregnation procedures of Bielschowsky-Maresch. Note the complex, net-like structure of the fibrinated wall. ×422.
- Fig. 22. Culture 5925-3. Another tubule from the same preparation shown in Fig. 21. A number of leukocytes have become incorporated in the fibrous wall. ×422.

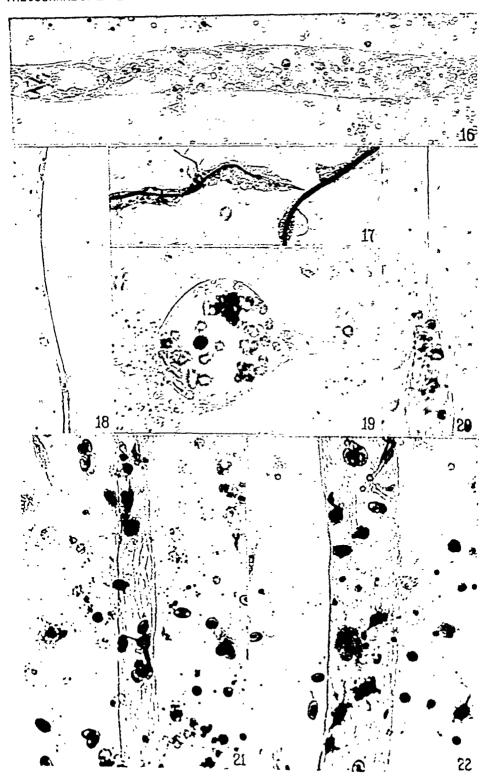








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# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

# IX. THE EFFECT OF AQUEOUS EXTRACTS OF CHICKEN TUMOR I ON YEAST NUCLEIC ACID\*

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A component of the chromosomes and therefore intimately associated with the rate and rhythm of cell reproduction, nucleic acid is an interesting substrate for extracts of tumor material. The disintegration of nucleic acid by such extracts has been reported by several investigators including Somekawa (1), who observed that aqueous extracts of Chicken Tumor I caused a separation of purine derivatives from yeast nucleic acid, and Edlbacher and Kutscher (2), who claimed that mammalian tumors split phosphoric acid from thymus nucleic acid whereas extracts from Chicken Tumor I had little if any effect of this kind.

A review on nucleic acids by Levene (3) led to the belief that these isolated observations, reported without tests for biological potency, might be correlated and extended, and to the opinion that an important structural characteristic of nucleic acid had been ignored; namely, that the arrangement of nitrogenous, sugar, and phosphoric acid groups is such that the ester bonds between component nucleotides must be broken to release more than one-quarter of its phosphoric acid and that they may be broken without release of inorganic phosphorus or of nitrogenous bases. This division of nucleic acid into its four nucleotides when the ester bonds are broken by enzyme action may be called polynucleotidase activity.<sup>1</sup>

<sup>\*</sup> This investigation was carried on under the Rutherford Denation.

I Levene and Dillon (4) use the same term for the release of an arbitrary amount of inorganic phosphorus in unit time from nucleic acid by an enzyme from intestinal juice.

The polynucleotidase and phosphatase activities of aqueous extracts of Chicken Tumor I are reported in this paper with evidence demonstrating a relationship between two systems, polynucleotidase activity and associated inhibiting material, and tumor-inducing agent and associated inhibiting material.

### Methods

As defined in the foregoing, polynucleotidase activity is the division of nucleic acid into its four component nucleotides and therefore the measurement of this activity depends on the separation of nucleic acid from nucleotides. This separation was accomplished by precipitating nucleic acid at its iso-electric point, pH less than 2.0, with a solution of 1.25 gm. uranyl chloride in 10 per cent trichloracetic acid. This mixture yields a supernatant solution of nucleotides and less complex derivatives of nucleic acid which can be separated from the precipitate of intact nucleic acid by spinning the mixture in a centrifuge (9).

As a source of such enzyme activity various products of Chicken Tumor I were tested. These products were Berkefeld filtrates of fresh tumor material, aqueous extracts of desiccated tumor material, and the supernatant fluid obtained by treating the aqueous extract with aluminum hydroxide Type C prepared as described by Willstätter and Kraut (5). The details of preparation of these products may be found in the papers of Murphy and his associates (6). All extracts used in the following experiments were prepared by extracting 1 gm. dry tumor powder with 60 cc. distilled water, separating the insoluble matter by spinning in a centrifuge at 1200 R.P.M. for 10 minutes, and decanting the supernatant soluble substances. The aluminum supernatant fluids were prepared by shaking a volume of aqueous extract with the gel from an equal volume of aluminum hydroxide Type C, spinning the mixture in a centrifuge at 1200 R.P.M. for 5 minutes, and decanting the supernatant fluid. This supernatant fluid is referred The concentration of chicken tumor substance in solution was determined by drying 2 cc. of solution under reduced pressure in a desiccator containing concentrated sulfuric acid until the weight of dried material was constant.

The disintegration of yeast nucleic acid was observed in mixtures containing 1 volume, 1 to 5 cc., of chicken tumor product, 3 volumes of yeast nucleic acid in 0.15 M buffer solution, and 1 volume of an aqueous solution of phenol. This mixture is called the digestion mixture throughout the paper. The buffers used for pH's of 5.0 to 7.0 were the sodium hydroxide-sodium citrate mixtures prepared according to Clark (7), and for pH's of 7.0 to 9.0 the sodium veronal-hydrochloric acid mixtures prepared according to Michaelis (8). The final concentration of nucleic acid was 1 to 4 per cent and of phenol was no greater than 0.3 per cent. The digestion mixtures, unboiled and boiled, were incubated in tubes with rubber stoppers at 37.5°C. until aliquots taken at regular intervals indicated that maxi-

mum disintegration of nucleic acid had been attained. Each aliquot was examined bacteriologically by smear and by subculture on agar because the final concentration of phenol was of low antiseptic power particularly for members of the aerobic, spore-bearing group of bacilli whose intensive disintegration of yeast nucleic acid is to be reported elsewhere with a more detailed description of the chemical methods (9).

The products of disintegration of nucleic acid were separated from intact nucleic acid by the following procedure. 4 cc. samples of the digestion mixtures were pipetted into centrifuge tubes of 50 cc. capacity and to each sample was added 11 cc. distilled water and 10 cc. of the precipitating reagent. The mixture was shaken vigorously for a few seconds and stirred thoroughly twice during a 10 minute interval. The white flocculent precipitate was separated from the yellow supernatant fluid by spinning the mixture in a centrifuge at 900 R.P.M. for 3 minutes. Then the supernatant fluid was decanted into a 50 cc. volumetric flask, and the precipitate was broken up with 10 cc. of precipitating solution previously diluted to 2/5 concentration with distilled water. Separation of the precipitate and supernatant was repeated twice as described and the three supernatants pooled and made up to 50 cc. with dilute precipitating reagent. The precipitate was dissolved in 25 cc. of N sodium carbonate.

In this way two solutions were made available for analysis of nitrogen and of phosphorus. Total nitrogen was determined by the micro-Kjeldahl method of Van Slyke (10); inorganic phosphorus was determined by the colorimetric method of Leiboff (11); and total phosphorus was determined by a modification of the method of Leiboff (9). From these analyses of the two solutions the polynucleotidase and phosphatase activities were calculated by the use of appropriate factors.

The estimations of enzyme activity were based on the structural formula of nucleic acid reported by Levene (3). Polynucleotidase activity was calculated, in terms of milligrams of nucleic acid disintegrated, by multiplying by 6.13 both the increase of total nitrogen soluble in the uranyl chloride supernatant fluid and the decrease of total nitrogen in the uranyl chloride precipitate, and by multiplying by 10.38 both the increase of total phosphorus in the uranyl chloride supernatant and the decrease of total phosphorus in the uranyl chloride precipitate. Similarly phosphatase activity was calculated, in terms of milligrams of nucleic acid disintegrated to release inorganic phosphorus, by multiplying by 10.38 the inorganic phosphorus soluble in the uranyl chloride supernatant fluid. The factor 6.13 is the ratio of the molecular weight of yeast nucleic acid to the weight of 15 atoms of nitrogen; i.e., 1287.5/210. The factor 10.38 is the ratio of the molecular weight of yeast nucleic acid to the weight of 4 atoms of phosphorus; i.e., 1287.5/124.1.

Except where stated each observation in the tables showing results is the difference of analysis of boiled digestion mixture from that of unboiled digestion mixture.

### RESULTS

Fifty aqueous extracts from different Chicken Tumor I powders have been investigated for hydrolysis of yeast nucleic acid: forty-four caused a disintegration of nucleic acid as considerable as that of chicken liver, kidney, spleen, and much more considerable than that of muscle. The other six extracts caused little or no disintegration of nucleic acid for reasons possibly associated with their pathological character; *i.e.*, the induction or inhibition of tumor growth.

Variations in the tumor-inducing power of different Chicken Tumor I powders have been noted by Murphy and Sturm (12), who classify the pathological activity of such powders as active, neutral, inhibiting: an active powder yields an aqueous extract which will induce an easily measurable tumor in 10 to 14 days after the extract is injected intracutaneously into the breasts of barred Plymouth Rock hens; an inhibiting powder, usually from a slow growing tumor, yields an aqueous extract which, when mixed with an active extract, prevents completely the appearance of a tumor for at least 5 weeks after injection; and a neutral powder, usually one which has been kept more than a year, neither induces nor inhibits tumor growth. The aluminum supernatant fluid when obtained from an active extract is called active Sa, when obtained from a neutral extract is called neutral Sa, and when obtained from an inhibiting extract is called inhibitor Sa. The aluminum supernatant fluids are either active or neutral pathologically but never inhibiting because the aluminum hydroxide gel adsorbs the inhibiting material to a greater degree than it does the tumor-inducing agent.

The foregoing definitions have been made to avoid confusion between enzyme and biological activities: in all cases the pathological term is given to the source of enzyme activity. Further aids to clear understanding of the tables are the summaries at the head of each table which give the final concentration of substrate, source of enzyme activity, and of antiseptic, and which give the pH, time of incubation at 37.5°C., and the results of subculture on agar slopes. Finally, under the heading "Nucleic acid disintegrated" the number (1) refers to nucleic acid calculated from the total nitrogen in the uranyl chloride supernatant fluid, the number (2) refers to nucleic acid calculated from the total phosphorus therein, the number (3) refers to nucleic acid calculated from the total nitrogen in the uranyl chloride precipitate, and the number (4) refers to the nucleic acid calculated from the total phosphorus therein.

Polynucleotidase Activity of Tumor-Inducing Extracts of Chicken Tumor I.—The polynucleotidase activity of active extracts of Chicken Tumor I caused a maximum disintegration of 44 per cent of yeast nucleic acid in 1 week at 37.5°C. at pH of 6.5. The enzyme activity

(as well as the tumor-inducing activity) was destroyed by heating the extracts at temperatures above 55°C. for 30 minutes. These statements are substantiated in Table I.

### TABLE I

Polynucleotidase Activity of Aqueous Extracts of Chicken Tumor I Powders

Nucleic acid, 1 per cent.

Chicken Tumor I, 0.22 per cent.

Phenol, 0.22 per cent.

pH, 6.5. Time of incubation, 1 week. Subcultures on agar, negative.

Source of enzyme activity	(A) Nucleic acid dis- integrated		(B) Nucleic acid left intact		(C) Hydrolysis
, , , , , , , , , , , , , , , , , , , ,	(1)	(2)	(3)	(4)	$C = \frac{A}{A + B} \times 100$
	rit.	T.L.	r:[.	mg.	per cent
Active extract	90.72	90.83	113.96	115.74	44
Active extract	90.11	90.82	115.06	116.77	44
Boiled extract	6.13	2.07	198.61	204.49	3
Boiled extract	5.52	2.07	199.22	203.45	3

### TABLE II

# Polynucleotidase Activities of Extracts of Chicken Tissues

Source of enzyme activity	Optimum pH	Nucleic and dis- integrated (2)	Nucleic seid dis- interrated per mg, dry weight of extract
		est.	mg.
Liver	8.8	62.80	3.49
Kidney	8.8	52,42	2.91
Chicken Tumor I	7.5	45.15	4.10
Spicen .	8.8	43.08	2.70
Muicle	70	5.19	0.65

Compared with those normal tissues of high polynucleotidase capacity Chicken Tumor I has an activity between that of kidney and that of spleen and much greater than that of muscle. In this experi-

ment all extracts of normal tissues were prepared as described for extracts of Chicken Tumor I and the dry weight per cubic centimeter of extract determined. The polynucleotidase activities of these extracts are given in Table II.

Consideration of Table II reveals that the unit activity per milligram dry weight was greater than that of any normal tissue investigated.

Differences between Polynucleotidase and Phosphatase Activities of Active Extracts of Chicken Tumor I.—The polynucleotidase and phos-

## TABLE III

Rates of Activity of the Polynucleotidase and Phosphatase of Chicken Tumor I Time of incubation, 4 weeks. Nucleic acid, 1 per cent.

pH, 6.5. Chicken Tumor I, 0.22 per cent.

Subcultures of agar, negative. Phenol, 0.22 per cent.

Phenol, 0.22 per				(C)
	(A Nucleic acid	disintegrated	(B) Nucleic acid dis- integrated to release inorganic phos-	Ratio of polynucleo tidase to phos- phatase
Time of incubation	(1)	(2)	phorus	C = A/B
days 0 1 2 5 7 14 21 28	mg. 5.50 26.36 44.75 76.13 90.72 91.95 93.79 93.79	mg. 4.88 26.16 43.70 75.77 90.82 93.42 94.15 94.15	ms. 2.07 6.54 12.14 24.39 32.59 41.52 48.89 49.30	2.5 4.0 3.6 3.1 2.8 2.3 1.9

phatase activities of extracts of Chicken Tumor I can be distinguished by the rate of hydrolysis, the effect of hydrogen ion concentration, and the effect of low oxygen tension.

The rate of hydrolysis for each enzyme activity at pH 6.5 is given in Table III, which shows that the polynucleotidase starts faster and ends sooner than the phosphatase, and that no more than one-third of the disintegrated nucleic acid released inorganic phosphorus.

The optimum pH for polynucleotidase activity is about 7.5, whereas it is about 6.5 for phosphatase activity, as shown in Table IV.

An environment of low oxygen tension was produced in a glass jar, containing the digestion mixtures in tubes without stoppers, after withdrawal of air, by restoring atmospheric pressure with hydrogen. In this environment the phosphatase activity was twice that in air whereas the polynucleotidase activity was the same as in air, as shown in Table V.

Phosphatase Activity of Tumor-Inducing Extracts of Chicken Tumor I.

—The phosphatase activity of active extracts of Chicken Tumor I

### TABLE IV

Effect of Hydrogen Ion Concentration on the Polynucleotidase and Phosphatase Activities of Chicken Tumor I

Nucleic acid, 2 per cent. Chicken Tumor I, 0.37 per cent. Phenol, 0.1 per cent. Time of incubation, 3 weeks. pH, as shown.
Subcultures on agar, negative.

ьн	Nucleic acid disintegrated		(B) Nucleic acid dis- integrated to release	(C) Ratio of polymucleo- tidast to phos-	
γ	(1)	(2)	inorganic phos- phorus	$\begin{array}{c} \text{phatase} \\ C = A/B \end{array}$	
	Fig.	77.[-	Fit.		
5.0	144.0	144.6	79.41	1.80	
5.5	150.0	149.7	80.96	1.85	
6.0	153.2	154.5	90.82	1.70	
6.5	159.0	157.5	99.13	1.59	
7.0		162.4	78.37	2.07	
7.5		171.2	39.44	4.35	
8.0		160.7	12.46	12.89	
8.5		152.4	8.30	18.40	
9.0		142.1	10.38	13.69	

caused a maximum disintegration of 15 per cent of the nucleic acid in the digestion mixture at pH of 6.5 in 3 weeks at 37.5°C. The inorganic phosphorus released from nucleic acid may have come from it directly or indirectly through nucleotides or through phosphoric acid ester of ribose as the immediate substrate. This alternative is preferred because there is a definite lag of the phosphatase behind the polynucleotidase activity. Therefore, the phosphatase activity is calculated in terms of percentage hydrolysis of disintegrated nucleic acid. So defined, the phosphatase activity of Chicken Tumor I is much less

intense than that of any extract of normal tissue investigated and it has an optimum pH different from that of the polynucleotidase. These statements are substantiated in Table VI.

### TABLE V

Polynucleotidase and Phosphatase Activities of Chicken Tumor I Extracts in an Environment of Air and of Hydrogen

Nucleic acid, 3.75 per cent. Chicken Tumor I, 0.30 per cent. Time of incubation, 1 week. pH, 7.3.

Phenol, 0.12 per cent.

Subcultures on agar, negative.

Atmospheric environment	(A) Nucleic acid disintegrated (2)	(B) Nucleic acid disintegrated to release inorganic phosphorus	(C) Polynucleotidase activity C = A/150	(D) Phosphatase activity D = B/150
	mg.	mg.	per cent	per cent
Hydrogen	71.8	22,0	47.9	14.4
Hydrogen		21.8	47.9	14.2
Hydrogen	71.8	22.0	47.9	14,2
Air	72.7	10.0	48.5	6.5
Air	71.8	11.2	47.9	7.3
Air	70.9	10.6	47.3	6.9

### TABLE VI

Phosphatase Activities of Extracts of Chicken Tissues

Nucleic acid, 3.75 per cent. Phenol, 0.1 per cent. Time of incubation, 3 weeks. pH, the optimum for each extract.

	Optimum pH	(A) Nucleic acid disintegrated (2)	(B) Nucleic acid disintegrated to release inorganic phosphorus	(C) Phosphatase activity $A = \frac{A}{B} \times 100$
		mg.	mg.	per cent
Liver	8.8	62.80	38.57	61
Kidney	8.8	52.42	32.49	62
Chicken Tumor I	6.5	40.16	13.39	33
Spleen	8.8	43.08	24.46	57
Muscle	7.0	5.19	4,15	80

Polynucleotidase and Phosphatase Activities of Sa from Active Extracts of Chicken Tumor I.—Adsorption of material from aqueous extracts

by aluminum hydroxide Type C in most cases increases the absolute intensity of polynucleotidase activity and always (twenty-five tests) increases the unit activity per milligram dry weight of Chicken Tumor I in solution. On the other hand, the absolute intensity of the phosphatase activity in all cases is decreased, and the unit activity increased though not to as great a degree as the polynucleotidase. The effect of adsorption with aluminum hydroxide Type C on the enzyme activities of the extracts is shown in Table VII. The frequent increase of

#### TABLE VII

Effect of Adsorption with Aluminum Hydroxide Type C on the Polynucleotidase and Phosphatase Activities of Extracts of Chicken Tumor I

Nucleic acid, 1.87 per cent. Chicken Tumor I in extract, 11.1 mg. per cc. Chicken Tumor I in Sa, 1.7 mg. per cc. Phenol, 0.1 per cent. Time of incubation, 3 weeks. pH, 6.5.

Chicken Tumor I in digestion mixture:

with extract.... 33.3 mg. with Sa..... 5.1 mg.

Source of enzyme activity	(A) Nucleic acid disinterrated (2)	(B) Nucleic acid disintegrated to release inor- ganic phosphorus	(C) Polynucleotidase activity per ms. Chicken Tumor I C = A/33.3 or A/5.1	(D) Phosphatase activity per me. Chicken Tumer I D = B/33.3 or B/5.1
	mt.	mg.	rig.	mr.
Extract	73.14	46.81	2.2	1.4
Extract	72.77	44.84	2.2	1.3
Sa	81.26	24.60	15.9	4.8
Sa	79.96	24.29	15.7	4.8

total activity caused by treating the extract with aluminum hydroxide gel suggested that some inhibiting substance had been removed by adsorption. Similar experiments by Murphy and associates (12) had disclosed the existence of material which inhibited the tumor agent, and for this reason the suggestive relationship between the two systems, polynucleotidase and associated inhibiting substance, and tumor agent and associated inhibiting substance, was tested more definitely.

Polynucleotidase Activities of Sa's of Graded Tumor-Inducing Power.

—Because the polynucleotidase activity caused a maximum disintegra-

tion of nucleic acid in 1 week and because the tumors reach a measurable size in 10 to 14 days it was possible to predict the relative size of tumors induced by Sa's of active, neutral, and inhibiting extracts. Within the error associated with the use of only two dimensions as accurate indices of the size of the tumors there was a rough proportionality between the polynucleotidase activity and the size of the tumors induced by these aluminum supernatant fluids. This propor-

### TABLE VIII

Polynucleotidase Activities of Sa's of Graded Tumor-Inducing Power Nucleic acid, 1.8 per cent. Phenol, 0.1 per cent. pH, 6.5.

Chicken Tumor I in active Sa, 1.7 mg. per cc. Chicken Tumor I in neutral Sa, 1.6 mg. per cc. Time of incubation, 3 weeks. Chicken Tumor I in inhibitor Sa, 0.9 mg. per cc. Subcultures on agar, negative.

Source of enzyme activity	(A) Nucleic acid disintegrated (2)	(B) Chicken Tumor I in digestion mixtures	(C) Nucleic acid disintegrated per mg. Chicken Tumor I C = A/B	(D) Maximum and minimum size of tumors (6 injections for each Sa)
	mg.	mg.	mg.	cm.
Active Sa	81.26	5.1	15.9	$2.8 \times 2.0$
Active Sa	79.96	5.1	15.7	to 2.6 x 2.1
Neutral Sa	17.85	4.8	3.7	0.7 x 0.5
Neutral Sa	17.27	4.8	3.6	$0.4 \times 0.3$
Inhibitor Sa	3.31 3.99	2.7 2.7	1.2 1.5	No growth

tionality is demonstrated in Table VIII, and was repeated in the only other experiment of this kind done.

Polynucleotidase Activity of an Active Sa When Mixed with Extracts of Graded Tumor-Inhibiting Power.—Extracts of graded tumor-inhibiing power were prepared from active, neutral, and inhibiting powers in the usual way. Each extract was heated to 55°C. for 30 minutes to destroy any polynucleotidase and tumor-inducing activity, cooled to room temperature, and mixed with an equal volume of an active Sa.

6 cc. of this mixture was tested for polynucleotidase activity with 14 cc. of 2.5 per cent yeast nucleic acid in buffer at pH of 6.5, and 0.4 cc. was injected intracutaneously into the breasts of barred Plymouth Rock hens. Both experiments performed in this way demonstrated, as in Table IX, a rough proportionality between inhibition of the tumor agent and inhibition of the enzyme activity.

### TABLE IX

Polynucleolidase Activity of an Active Sa When Mixed with Equal Volumes of Extracts of Graded Tumor-Inducing Power

Nucleic acid, 1.75 per cent. Phenol, 0.22 per cent. pH, 6.5. Time of incubation, 3 weeks. Subcultures on agar, negative.

Pathological character of extracts mixed with active Sa	(A) Nucleic acid disintegrated. Extract heated to 55°C. (2)	(B) Nucleic scid disintegrated. Extract boiled (2)	(C) Inhibition of polynucleotidase activity $C = \frac{B - A}{B} \times 100$	Maximum and minimum size of tumors (6 injections for each mixture of extract and Sa)
	TIE.	rig.	fer cent	ers.
Active extract	24.13	25.09	3.8	1.7 x 1.6
			•	to
				1.5 x 1.5
Neutral extract	14.82	24.64	40.0	0.9 x 0.8
			•	0.8 x 0.8
Inhibiting extract	5.75	25.62	77.6	No growth
Water	25.70	25.70	0	1.8 x 1.6
			;	to :
	1	1	,	, 1.6 x 1.6

### DISCUSSION

The results reported in this paper confirm the observations of Edlbacher and Kutscher that extracts of Chicken Tumor I have very little phosphatase activity at pH's greater than 8.0 and are consistent with the report of Somekawa that these extracts cause a separation of purine substance from yeast nucleic acid more considerable than that of muscle. All these results may be correlated by a tentative explanation that under the influence of enzymes in Chicken Tumor I yeast nucleic acid undergoes a progressive disintegration through the stage of nucleotides to phosphoric acid ester of ribose and free nitrogenous bases.

The pathological significance of the polynucleotidase in Chicken Tumor I products cannot be stated definitely, though the rough proportionality between the enzyme system and the pathological system, the occurrence of the optimum pH in the physiologically important zone of pH 7.0 to 7.8, and the similarity of the minimal lethal temperature lead to the opinion that the relationship is not haphazard.

### SUMMARY

- 1. Aqueous extracts of Chicken Tumor I cause a disintegration of yeast nucleic acid.
- 2. Two enzyme activities, a polynucleotidase and a phosphatase, are demonstrated.
- 3. These activities are distinguished by different reactions to an environment of hydrogen, different optima of hydrogen ion concentration, and different rates of activity.
- 4. Adsorption with aluminum hydroxide Type C removes substances inhibiting the polynucleotidase activity.
- 5. There is a rough proportionality between the polynucleotidase activity and the tumor-inducing power of different fractions of extracts of Chicken Tumor I.
- 6. There is a rough proportionality between the inhibition of polynucleotidase activity and the inhibition of the tumor agent.
- 7. The polynucleotidase activity of Chicken Tumor I is as great as that of spleen, slightly less than that of kidney and liver, but much greater than that of muscle. There are also qualitative differences.

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# STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF RABBITS

### II. BACTERIOLOGICAL INVESTIGATIONS

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Plates 16 and 17

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Spontaneous conjunctival folliculosis, a disease widespread among rabbits, has been described in a preceding article (1) as occurring in two clinical forms, Type I and Type II. The former is characterized by a dormant chronicity with discrete lesions, whereas the second type, a more actively progressive disease, presents an inflammatory follicular reaction involving all the palpebral conjunctival surfaces. It was found that the transition from one type to the other, an infrequent occurrence in nature, could more often be induced artificially by experimental procedures. From our studies a conclusion was drawn that the causal factor of the affection is not an ultramicroscopic virus but an infectious agent behaving after the manner of bacteria of low grade pathogenicity.

In this second paper the results of our bacteriological investigations will be presented.

### Methods

The methods employed in cultivation tests were similar, with the exceptions to be noted, to those in bacteriological studies on spontaneous monkey folliculosis (2). The exceptions consisted in the use of: (a) defibrinated rabbit blood instead of horse blood in the preparation of the blood agar medium, a substitution made merely for the sake of convenience; (t) dextrose instead of five carbohydrates, of which a 5 per cent solution was filtered through a Berkefeld V candle, and 5 cc. of the sterile filtrate were added to 95 cc. of the blood agar; (c) agar containing 2 per cent Witte's peptone, adjusted to pH 7.4, cooled to 45°C., and into this sterile, defibrinated, rabbit blood was introduced to make 8 per cent by volume.

Although plain dextrose agar readily supported the growth of the several species of bacteria that were cultivated, the employment of blood agar was found to be preferable, especially for initial cultures, in that it afforded a more profuse growth of fragile organisms and facilitated their recovery. For each test six plates of blood agar and four tubes of leptospira medium, prepared as already described (2), were seeded with saline solution suspensions of excised conjunctival tissue. The excised tissue, rather than expressed follicular, or grattage material, was found to be more advantageous for bacterial cultivation.

# Cultures of Affected Tissue and Control Materials

The bacterial flora of the conjunctival tissue obtained from normal rabbits was not unlike that from monkeys (2). Staphylococci and diphtheroids predominated, whereas the microorganisms less frequently encountered were the Gram-negative, motile or non-motile, chromogenic or non-chromogenic bacilli of indeterminate species; organisms of the subtilis group; yeasts and moulds. The relative incidence of these bacteria in normal tissue was low, and in affected conjunctivae much higher. Bacterium granulosis and Bacterium simiae, associated with human or experimental trachoma (3), and with simian folliculosis (2), respectively, were not found. Nor could we isolate Bacterium monocytogenes (3, 4), the incitant of fatal epizootics among rabbit breeding stocks.

Thirty-eight strains of the indeterminate group of organisms were collected, and of these, sixteen different cultures were injected into 46 rabbits in the manner already described (1). It should be noted that characteristic folliculosis was not induced thereby, although in some instances transitory, diffuse abscess formation resulted and in others localized pustules at the points of scarification. In all cases healing was prompt and normal conditions were restored within a week or two after inoculation.

The different species of ordinary or indeterminate bacteria, as mentioned, were not the only ones which were isolated from the rabbit folliculosis tissue. In addition to these, we have cultivated from affected conjunctivae a new species of microorganism which, after inoculation into rabbits, induced a conjunctival follicular reaction indistinguishable from the spontaneous disease as it occurs in nature. This special bacterium has been isolated from seventeen of 24 rabbits

<sup>&</sup>lt;sup>1</sup> All operations on animals were performed with the aid of ether anesthesia.

showing fully developed, diffuse (Type II) folliculosis. There was, however, considerable difficulty in cultivating it from animals having the Type I disease, which is less readily transmissible to normal rabbits (1), and from those showing a mild degree of the diffuse form of the affection. For example, only five positive cultures were obtained from eighteen such animals.

# Description of the Microorganism

In the following brief form will be presented the chief morphological and biological characteristics of the special microorganism isolated and cultivated from conjunctival folliculosis of rabbits.

Morphology.—The common form of the microorganism is a slender, thin bacillus, with pointed ends, two or three times longer than its width, and ordinarily smaller in size than Bacterium granulosis or simiae, or 0.2 to  $0.3\mu$  wide and 0.5 to  $1\mu$  long. The appearance of the minute, delicate, tenuous bacilli in stained film preparations is not unlike that of H. influenzae (Fig. 1). It is pleomorphic; one often sees shorter coccoid or lanceolate structures frequently occurring in pairs, placed end to end. As a rule, smaller organisms preponderate in growths in leptospira medium and larger ones on agar.

Staining Reactions.—The organisms are Gram-negative and non-acid-fast. With stains for capsules, especially those of Muir or Casares-Gil, a faint, delicate envelope can be observed outside of a thin halo surrounding the bacilli or the diplo-lanceolate forms. The capsule is of much finer texture than the membranes surrounding the granulosis or simiae species, and it is not demonstrable by ordinary tinctorial methods. The Casares-Gil stain also reveals peritrichal flagella which apparently proceed from the capsular material (5). One flagellum is attached to each pole and one, two, or three, usually two, to each side. The thready structures are long, tenuous, and wavy and in stained film preparations often appear matted or thrown to one side (Fig. 2).

Metility.—The multiple flagella contribute to the peculiar and highly active motility of the organism. The bacilli move rapidly in a circular or linear direction with a motion that may be either waddling or tumbling. There are some, however, that revolve about their central points as axes, like a pinwheel, without forward progression. The motility is not lost with the age of a culture, nor is it influenced by the medium employed.

Spores and metachromatic granules are absent.

Agar Plates.—The colonies first appear in 24 to 48 hours as minute, smooth, spherical growths about 1 mm, in diameter, enlarging in 24 to 48 hours to a rire of 2 or 3 mm. They are translucent, somewhat convex, moist, and mucoid. The edge is entire; the substance is slightly greyish and homogeneous. There is a ten lergy of one colony to orallow with its neighboring one (Fig. 3). When many

nified at 10 diameters as with a colony microscope, it exhibits a peaked center and a sharply defined, but slightly raised edge (Figs. 4 and 5). The growth on blood agar is more profuse and the colonies are somewhat less translucent and more greyish. As occurs with granulosis and simiac microorganisms, cultures of affected conjunctival tissue yield only an occasional colony of the special bacterium among many miscellaneous growths.<sup>2</sup> The colonies are sometimes adherent to, or incorporated within an alien one. In the latter instances, mixed growths can be purified by repeated subplants on fresh media.

Agar slants exhibit within 48 hours a slightly greyish, translucent, coalescent growth, which is glistening, mucoid, homogeneous, and non-spreading. The water of syncresis appears uniformly cloudy or milky, depending on the amount of growth.

Leptospira Medium.—24 hours after inoculation, a faint nebulosity occurs just at the surface. Following this there is an ingrowing, sac-like mass, with its base, 5 mm. across, lying at the center of the under surface and extending for 5 mm. into the medium. The area spreads out laterally so that within a day or two there is a uniformly opaque, whitish layer up to 1 cm. high. A slowly progressive extension of the clouding takes place until the bottom of the tube is reached, usually within 7 days.

Gelatin.—No liquefaction occurs. In stab cultures one observes a tenuous, arborescent, non-spreading line of growth. Colonies on gelatin agar appear greyish, mucoid, and confluent but they are less numerous than on blood agar.

Broth.—In this medium the growth is uniformly turbid without pellicle formation.

Litmus Milk.-Unchanged.

Potato.—Faint, buff colored (changing to brown after 5 days), non-spreading, sparse surface culture.

Indol.-Not produced.

<sup>&</sup>lt;sup>2</sup> A probable explanation for the sparse colony distribution of the three species of organisms in initial cultures of affected tissues is that the medium may not be completely satisfactory for primary isolations, or the organisms may be suppressed by the growths of the rapidly developing, concomitant, miscellaneous bacteria present. Indeed, it has been shown by Weiss that the growth of staphylococci per se chiefly by lowering the pH of the medium definitely inhibits the development of Bacterium granulosis—the degree of inhibition being measurable on a quantitative basis. Diphtheroids also suppress granulosis growth but not as markedly (6). As already indicated, staphylococci and diphtheroids are predominating organisms in cultures of affected conjunctival tissues. We have also observed the inhibition, under these conditions, of growths of granulosis, simiae, and the special rabbit bacteria. (See also Reference 7; and for a recent discussion of the principles underlying bacterial antagonism, Neufeld, F., and Kuhn H., Z. Hyg. u. Infektion-skrankh., 1934, 116, 95.)

Nitrates.-Not reduced.

Carbohydrate Reactions.—This organism is non-fermenting in type. No acid or gas is produced on dextrose, levulose, mannose, mannitol, saccharose, raffinose, inulin, galactose, maltose, salicin, xylose, dextrin, arabinose, amygdalin, lactose, dulcitol, rhamnose, trehalose, sorbitol, or inositol.

Other Properties.—The bacterium is aerobic and facultatively anaerobic. It produces no characteristic odor in cultures and is bile-resistant. The optimum temperature for development is 28–30°C. The thermal death point is at 56°C. for 15 to 30 minutes.

Filtrability.—Cultures suspended in saline solution failed, in ten tests, to pass through single disc Seitz and Berkefeld V filters. Similar material suspended, however, in hormone broth was filtrable through three of the four Berkefeld V filters used but not through Seitz discs. Precisely similar results were obtained when cultures of Serratia marcescens (Bacillus prodigiosus) were employed. The filtrability of many species of bacteria is possible in a hormone broth suspension and the results of tests with the special bacterium do not necessarily indicate that it has a filtrable stage or that it is a "filtrable" organism. However that may be, we made use of this as a practical finding in selective filtration through pervious Berkefeld V candles of mixtures of the bacterium with larger, non-filtrable organisms, such as staphylococci, so as to facilitate its recovery in a pure state from initial mixed cultures. The methods followed have already been described in similar experiments with Bacterium granulosis (7).

Serological Reactions.—Repeated intravenous injections into rabbits of living cultures of the special bacterium yielded antisera with an agglutinin titer of 1:1000 to 1:20,000 for all the available cultures, 22 in number, of the microorganism. The specificity of the agglutination was shown by the negative reactions in 1:10 dilution of the sera in tests with ordinary bacteria and with 37 strains of the miscellaneous, indeterminate bacteria recovered from rabbit conjunctivae. At this point mention should be made of the fact that Bacterium granulosis and simiae exhibit no cross-agglutination with the special bacterium. Hence the rabbit organism is serologically specific.

No agglutinins against this bacterium were found in 1:2 to 1:15 dilutions of the sera obtained from 29 normal rabbits. On the other hand, thirteen of fourteen animals infected by subconjunctival inoculation of the organism, as will be immediately described, revealed specific clumping in 1:20 to 1:160 dilutions of serum in tests made during the 3rd to 6th week after inoculation. Nevertheless, the organism was not agglutinated by the serum of rabbits having spontaneous folliculosis or the experimental disease evoked by injection of affected tissue. By comparison, no agglutinins were found in trachoma serum against Bacterium granulosis (8), nor in monkey folliculosis blood against Bacterium simiae (2). Moreover, Weiss (9), in a summary of his own and prior reports on localized infections of the conjunctiva, a class that includes rabbit folliculosis, has concluded that such affections do not induce appreciable antibody formation.

Thus in the general properties as given, the bacterium isolated and cultivated from cases of rabbit folliculosis appears to be distinct from other described microorganisms. It has, however, the same generic characteristics as *Bacterium granulosis* and *Bacterium simiae*. The diagnosis, or characters, of this genus may be stated as follows:

Small, slender Gram-negative rods present in the conjunctiva of man and animals affected by a follicular type of disease; mucoid type of growth which in early subplants takes place with some difficulty in ordinary media; motile, flagellated, and encapsulated; aerobic and facultatively anaerobic, produce no endospores, optimum temperature for growth 28–30°C.

On the other hand, the rabbit microorganism differs in specific characters from those of either the human or simian bacterium, in the degree of motility; in the arrangement of flagella; in the manner of growth on the same medium; in the reactions with carbohydrates, and especially in serological specificity, so that the designation of the rabbit bacillus as a distinct species is indicated.

# Pathogenicity Experiments

Sixteen cultures of the special bacterium were available for pathogenicity tests.

Of the sixteen cultures, fourteen individually and two pooled were injected into anesthetized animals. The 48 hour growth on agar slants of a second, or more remote generation, was suspended in 1 cc. of 0.9 per cent saline solution. 0.2 cc. of the suspension was introduced subconjunctivally into young rabbits having smooth lids. These animals were selected and quarantined in the manner already described (1). Only the left lower membrane received the culture and two rabbits were employed for each strain; hence 30 rabbits were employed for the sixteen cultures.

Only one strain was inactive; the remaining fifteen induced conjunctival follicular reactions in all but two of 28 inoculated animals. The resultant experimental disease could not be differentiated either clinically or histopathologically from the natural affection or from the infection evoked by inoculation of folliculosis tissue itself (1). In all instances the disease developed eventually into diffuse, Type II folliculosis (Figs. 6 to 8) and from six of such animals the identical organism was recovered. These latter strains were again shown to be specifically active in normal rabbits.

Instillation of cultures of the special bacterium into the conjunctival sac of normal rabbits, in accordance with the method used previously (1), also brought about the same type of specific infection in two of three animals. These two exhibited characteristic folliculosis, 11 and 18 days respectively after the last instillation. Furthermore, the same microorganism was recovered from the conjunctivae of both, in the 3rd and 7th week of their illness, when folliculosis was fully established.

Microorganisms in Tissues.—The stained sections of lesions induced by the special bacterium and film preparations of expressed follicular material were studied. In the latter instance, the Giemsa- or Gramstained grattage material obtained from 22 rabbits showed in ten of them miscellaneous bacteria varying in number and consisting chiefly of staphylococci and diphtheroids. In addition, in all the specimens were observed discrete or clumped, free or intracellular, Gram-negative bacteria which closely resembled the special microorganism in morphology (Fig. 9). An examination of sections of over twenty different follicular conjunctivae, stained by the Giemsa, Gram, or eosin-methylene blue methods, yielded similar results (Figs. 10 and 11). The microorganism, however, was found in such material to be smaller and often intracellular. It is of interest that the bacteria were seen in section or film preparations that were derived from rabbits having either the natural disease, or experimental folliculosis induced by inoculation of the special bacterium and of conjunctival tissue. Finally, cellular inclusion bodies that are characteristically associated with the action of many ultramicroscopic viruses were not observed.

### SUMMARY AND CONCLUSIONS

Before summarizing the experimental results presented herewith, a brief review is indicated of our study on follicular conjunctivitides existing in the different species of animals thus far examined.

Spontaneous follicular conjunctivitis of monkeys and chimpanzees, which resembles the early, or follicular, stages of human trachoma, has been shown to be an infectious disease, and to arise from the action of a bacterium and not an ultramicroscopic virus (2, 10). A new species of organism, designated tentatively as Bacterium simiae, n.sp., and capable of reproducing granular lids in normal monkeys and apes.

has been recovered from cultures of simian folliculosis tissues. This organism, although of a different species, is of the same genus as *Bacterium granulosis* which is associated with human trachoma (3).

The studies were extended beyond folliculosis in monkeys and apes to a similar disease widespread among stock rabbits. Its clinical appearance and microscopic changes resemble the spontaneous malady in similars with some minor differences (1). Like the latter, rabbit folliculosis was also shown to be infectious and contagious.

In the present paper a bacteriological investigation of the rabbit affection is described. In the experiments undertaken we followed the method used by Noguchi in his studies on trachoma, that is, the different organisms recovered from normal and affected conjunctivae were inoculated into the conjunctivae of selected, quarantined rabbits.

Among the bacteria thus isolated from folliculosis tissue a new species was disclosed that brought about conjunctival reactions apparently indistinguishable either from the disease seen in nature or from the experimental infection induced by inoculation of folliculosis tissue. The organism was specifically active in rabbits whether injected subconjunctivally or instilled into the conjunctival sac. Furthermore, the special bacterium has thus far been cultivated only from folliculosis cases and not from other forms of conjunctivitis nor from normal tissues. Finally, specifically pathogenic strains of the identical microorganism have been recovered from rabbits experimentally infected with the bacterium.

A distinctive feature of this bacterium is that it is of the same genus as the microorganisms heretofore designated as Bacterium granulosis and Bacterium simiae. It is clearly evident then that the three bacteria cultivated to this time form a new genus, for which we propose the name Noguchia. The diagnosis of the genus has already been described in this paper, but as was also shown in a foregoing section, certain specific characters differentiate the three. Accordingly, we suggest a change in the names of the granulosis and simiae species to the proposed binomials, Noguchia granulosis, n.sp., and Noguchia simiae, n.sp. The specific organism, having an evident causal relationship to rabbit folliculosis, can therefore be placed in this classification, with the name of Noguchia cuniculi, n.sp.<sup>3</sup>

<sup>3</sup> The classification of genus and species follows the recommendations of the Society of American Bacteriologists quoted by Bergey, D. H., in Manual of

In conclusion it would appear that in three types, at least, of follicular reactions in the conjunctiva—in man (the primary lesion of trachoma consisting essentially of follicles), in the simian, and in the rabbit—there is an intimate association of microorganisms having the same generic, but different specific properties.

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### EXPLANATION OF PLATES

### PLATE 16

- Fig. 1. Gram's stain. Agar slant culture. ×1000.
- Fig. 2. Casares-Gil stain, showing flagella; the staining fluid has thrown the structures to one side of the bacterium. ×1000.
  - Fig. 3. 48 hour growth on blood agar plate. Natural size.
  - Fig. 4. Same colonies photographed by reflected light. ×10.
  - Fig. 5. Same colonies photographed by transmitted light.  $\times 10$ .
- Fig. 6. Upper and lower conjunctivae of rabbit inoculated with a culture of Neguchia cuniculi. The characteristic folliculosis thus brought about should be compared with the condition seen in nature, as described and illustrated in the preceding paper (1). Natural size.

### PLATE 17

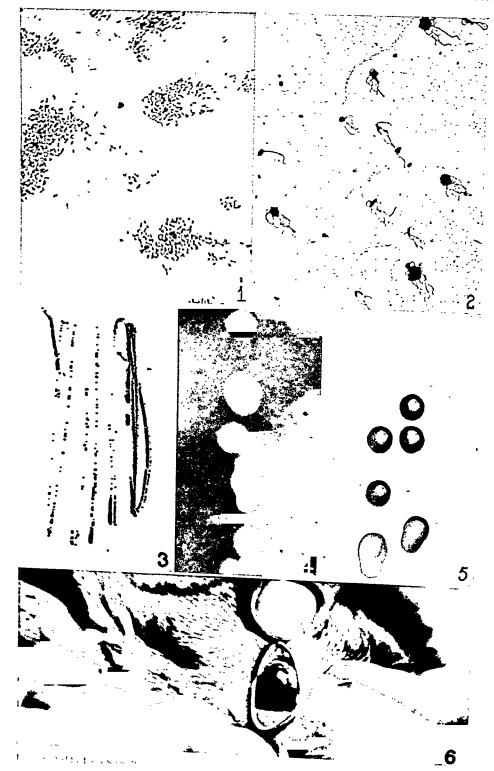
Fig. 7. Eosin and methylene blue stain. Conjunctiva obtained from a rabbit inoculated subconjunctivally with the microorganism. The induced lesions are

determinative bacteriology, and by Buchanan, R. E., in General systematic bacteriology, both published by Williams & Wilkins Co., Baltimore, 1925; also the Code of the Nomenclature Committee of the International Society of Microbiology (1930) quoted in Bergey's Manual, as stated, 1934, 4th edition, p. 23.

identical with those found in the natural disease or the experimental infection caused by injection of affected tissue, as illustrated in Paper I (1). ×122.

- Fig. 8. Eosin and methylene blue stain. Same as Fig. 7. Represents only a part, about a third, of the entire follicle. ×300.
- Fig. 9. Giemsa stain. Film preparation of conjunctival grattage material obtained from a rabbit having spontaneous folliculosis. Among miscellaneous microorganisms are seen bacteria with the morphology of *Noguchia cuniculi*. ×1000.
- Fig. 10. Eosin and methylene blue stain. Tissue section of spontaneous conjunctival folliculosis in a stock rabbit showing a clump of similar bacteria within a cell. ×1000.

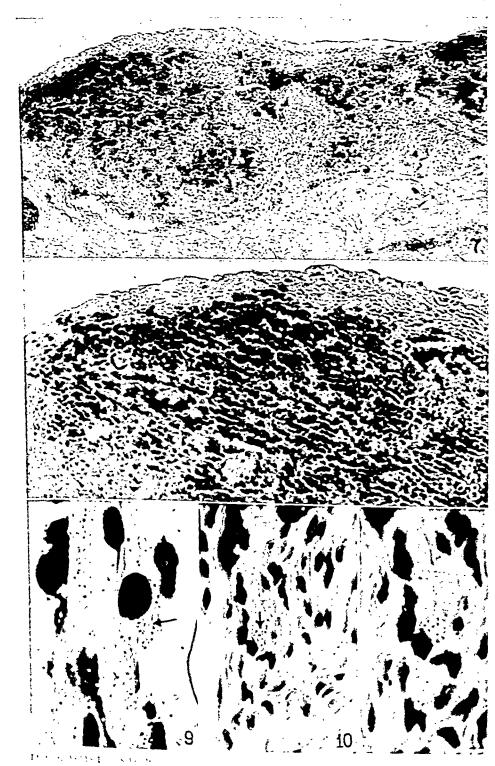
Fig. 11. Same. ×1500.



identical with the caused by injecting Fig. 8. Eosing part, about a thing Fig. 9. Giem obtained from a microorganisms ×1000.

Fig. 10. Eo conjunctival fo within a cell.

Fig. 11. Sai





# BACTERIOLOGICAL STUDIES ON AN EPIZOOTIC OF INTES-TINAL DISEASE IN SUCKLING AND NEWLY WEANED MICE

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In August, 1933, a spontaneous epizootic, with clinical signs of an acute intestinal affection, first appeared among the mice in the Rockefeller Institute breeding stock.<sup>1</sup> Previous epizootics caused by Salmonella infection have occurred at the Institute and have been described by Flexner (1) and his associates, Lynch (2), Amoss (3, 4), Webster (5, 6), and Amoss and Haselbauer (7). The present affection, however, was distinctive in its sharp limitation to suckling and newly weaned mice, as well as in difference in the stock affected, symptomatology, pathological changes, and in basic etiological origin. Epizootics similarly confined to the corresponding age group of animals and comparable in some other respects to the outbreak studied by us have previously been described (8–14).

For example, natural infection of hogs with Salmonella suipestifer is restricted to the very young, usually suckling or newly weaned shoats. "All authorities appear to agree that such infection does not occur naturally except in very young subjects" (8). When older animals present similar symptoms the disease is usually the virus affection known as hog cholera or swine fever. The fact that there is an independent, but similar, affection due to Salmonella infection would support the early investigations of Salmon and Theobald Smith (1885), and Welch (9) in their studies on hog cholera bacillus (8).

In this paper we shall record the clinical and pathological appearance of the disease in mice, its epizoology, and the results of bacteriological studies.

<sup>1</sup> The mice raised in The Rockefeller Institute for experimental purposes consist of two strains: an albino strain inbred for 22 years, known as the Rockefeller Institute strain, and a strain of Swiss mice. The former is bred and used in greater numbers than the latter.

# Clinical Signs

The malady was confined to suckling and newly weaned mice 7 to 24 days old. There was profuse diarrhea attended by apparent tenesmus and marked inanition. Loss of weight and tone was extremely rapid; complete prostration occurred within 24 to 72 hours after the initial signs and death took place in about one-third of the animals affected. Obstipation and cessation of suckling sometimes supervened, leading to a dehydration that was almost uniformly fatal. The obstipation was noted in about one-third of the mice. In the small number that recovered after showing this complication, necrosis of the tissues of the perianal region with sloughing frequently occurred. In the absence of obstipation, recovery was usually rapid and complete; after several days the recovered mice were indistinguishable from unaffected litter mates.

# Pathology

The gross lesions are limited largely to the intestinal tract and vary from slight hyperemia to frank necrosis of the lower ileum and colon. The spleen is dark red and contracted; the hyperemic tumefaction so frequently seen in mouse typhoid is not present. On the other hand, in recovered mice the spleen may show gross cicatrization.

Microscopically, the most characteristic changes are found in the lower ileum and colon. The mucosa is infiltrated with polymorphonuclear and red cells; it may exhibit generalized ulceration. The mucosa is often found as a slough in the lumen of the gut. The lymphoid tissue is increased, with hypertrophy of Peyer's patches particularly marked. The involvement of the gut is generalized and is not restricted to circumscribed ulcerations.

The spleen is hemorrhagic and contains many clasmatocytes. The pulp cells show varying stages of necrosis. A diffuse, parenchymatous degeneration of the liver occurs in varying degree, being somewhat more pronounced in the periportal areas. In marked cases there is a fatty degeneration of the liver parenchyma associated with localized hemorrhages. Only exceptionally does one observe focal necrosis of groups of liver cells. The kidneys reveal congestion and localized hemorrhages with early glomerular changes and granular degeneration of the tubular epithelial cells. An occasional, minute, focal hemorrhage is usually found in the brain and with this exception the organ appears unchanged.

In general the lesions consist of ulcerative enteritis; hemorrhagic and necrotic splenitis; fatty degeneration of the liver, and acute parenchymatous nephritis.

# Epizoology

Epizoological observations were largely limited to the time during which the experimental work was in progress. The caretakers of the

breeding room, however, had kept a record of the number of infected mice discarded prior to the time our investigations began, thus enabling us to estimate closely the number of animals affected.

The disease, as previously mentioned, was confined to infant mice, 7 to 24 days old. It quickly spread in sporadic outbursts throughout the entire colony, with a somewhat higher morbidity among the Swiss than the Institute strain. From 15 to 100 per cent of an individual litter would become sick; the average morbidity approximated 75 per cent, and of these 60 per cent died. In any cage litter mates that did not show signs of the illness within the first 48 hours after onset would invariably maintain their resistance completely. Their sleek, healthy, general appearance and activity were in striking contrast to those of their sick or recovering siblings.

The epizoological curve in this disease was directly comparable to that described by Lynch (2). The peak was in September, at which time the case incidence reached 35 per cent of an infant population of 4,000. Following this, the incidence gradually receded to a low point in December and January, at which time 11 per cent of 2,900 were ill. A secondary rise in February to 35 per cent of 3,800 infant mice was then observed with a subsequent abatement that is again approaching the previous low level at the time of writing. The occurrence of the two points of highest case incidence, namely, September and February, corresponds to those found by Pritchett (15) in her studies on the seasonal variation in the susceptibility of five strains of mice to infection per os with B. pestis caviac, a causal agent of mouse typhoid.

## Preliminary Investigations

Investigations were first undertaken to determine possible etiological factors.

Dict.—The routine diet used in the mouse room throughout the past 15 years consists of a teaspoonful of stale white bread soaked in fresh, pasteurized, Grade B milk for each mouse daily, and is supplemented by a mixture of equal parts of natmeal and buckwheat twice a week and by dog biscuit once a week. Substitutes for the various components of this diet, as well as substitutions of entirely different diets under well controlled conditions, were without effect in reducing the incidence of the disease, except in one particular, the details of which follow.

The extent of the morbidity and mortality was found to be dependent to a greater or less degree on the amount of water imbibed by pregnant females.<sup>2</sup> This is illustrated by the following protocol.

	No. of pregnant mice	No. of mothers showing infected litters	No. of young affected	No. of affected young that died
Given water with diet Without water		5 6	14 20	1 11

On the other hand, the epizootic continued when water was plentifully supplied to nursing mothers, so that the absence of this factor can be considered only as contributing to the gravity of the disease.

Contact Infection.—The infectious nature of the disease was determined by contact experiments in which sick mice were placed among normal, quarantined mice.<sup>3</sup> In four such experiments, in which 24 normal mice were used, 30 per cent of them developed the characteristic enteritis. Thus the resultant morbidity in the individual litters approximated that found in the epizootic as it occurred in the breeding room.

# Virus.—A virus as the cause was eliminated as follows:

Repeated filtration tests were made: the filtrates of viscera, brain, and intestinal contents, as well as emulsions of spleen, liver, and brain subsequently found to be bacteriologically sterile were inoculated by intraperitoneal, intracerebral, subcutaneous, and intramuscular routes without apparent effect. Furthermore, there was an absence, in especially prepared and stained sections of affected tissue, of specific cytotropism or inclusion bodies, such as are commonly encountered in many ultramicroscopic diseases.

*Protozoa*.—Repeated stool examinations and a study of pathological sections of the intestines and liver excluded a protozoal infection.

Bacteriological studies now became more promising and it is to this aspect of the problem that the remainder of this paper will be devoted.

<sup>2</sup> We are indebted to Dr. L. Whitman of the Rockefeller Foundation for his helpful suggestions as concerns this experiment.

<sup>3</sup> Impregnated Swiss females were obtained from an outside source and kept in rigid isolation in a room apart from those used for experimentation. They and their offspring were employed in this and in the other experiments to be described.

## Bacteriological Methods

Heart's blood, emulsions of spleen, liver, kidney, and brain, and contents of the different divisions of the large and small gut were used as the inocula for bacteriological cultures. The media employed consisted of blood agar and Endo plates, a modification (16) of the leptospira media of Noguchi, and hormone broth.

## Results of Cultivation Tests

Twenty-six mice showing typical signs of infection were used for cultivation purposes. From twenty, or 77 per cent, of these an organism was recovered the major characteristics of which definitely place it in the genus Salmonella. This organism was isolated from the intestine in every instance, and also from the heart's blood on three occasions, and the liver and spleen, respectively, on four occasions. No other species of organism was recovered from the liver, spleen, or heart's blood.

The number of colonies on Endo plates was highly variable, ranging from one to two on several plates inoculated with material from the same mouse to almost pure cultures. It was observed that the colonies were more numerous and predominated over those of other bacteria at the height of the epizootic.

Similar cultivation tests carried out on mothers of infected litters, including the mammary glands and excreta, yielded entirely negative results.

## Description of the Microorganism Recovered

Morphology and Staining Reactions.—It is a non-spore-forming, small bacillus 1 to  $3\mu$  in length and  $0.4\mu$  to  $0.7\mu$  in diameter. It is readily stained, Gram-negative, and although it usually appears discrete it may be seen in very short chains.

Growth Characteristics.—A variety of media readily supports its growth. Plain broth and agar slants, pH 7.4, show an abundant growth in 18 hours. Gelatin is not liquefied. Indol is formed. There is slight acidification but no coagulation of litmus milk. Nitrates are reduced to nitrites.

In carbohydrate broth no acid or gas is formed in lactose, saccharose, raffinose, inulin, salicin, dextrin, amygdalin, or inositol. Acid and gas are formed in glucose, levulose, mannose, galactose, arabinose, rhamnose, maltose, xylose, trehalose, and sorbitol. The reactions in mannitol and dulcitol were slightly irregular with only an exceptional culture not forming acid and gas. Like other Salmonella, the special organism produces no clouding of lactose broth in the closed branch of the fermentation tube devised by Theobald Smith. The hydrogen sulfide and the Voges-Proskauer reactions are negative.

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Serological Reactions.—Rabbit antisera were obtained that had an agglutination titer up to 1:12,800 for thirteen strains of the bacterium. The remaining seven strains recovered were all a variant that did not give the same agglutination titer but did conform in respect to sugar reactions, production of indol, and effect on litmus milk. Such serological variants are known to occur in natural or induced Salmonella infections in mice (4, 17, 18, 19). Rabbit antisera prepared from the variant organism showed agglutination in a dilution of 1:80 with the type bacterium.

Specific agglutinins were demonstrated in serum from each of 23, or 34 per cent, of 67 recovered mice with two having an agglutination titer of 1:640, three 1:160, five 1:80, four 1:40, eight 1:20, and one 1:10. Agglutinins for the variant were present in the same percentage of recovered mice. Agglutinins were also demonstrated in the serum of mothers having litters 100 per cent affected, in that two out of twelve gave agglutination in titers of 1:40 and 1:320.

It is noteworthy that agglutinins were not present in serum from normal quarantined mice; none of 50 showed agglutination.<sup>4</sup> As a further control, a duplicate set-up was made using the mouse typhoid organism, M.T. II (20) or Salmonella aertrycke, with which entirely negative results were obtained with individual sera from 27 recovered mice, and from seven mothers having infected litters.

A comparison with results obtained by other investigators in the related mouse typhoid epizootics is of interest. For example, Amoss (4) found that 22 of 56 survivors showed complete agglutination with Salmonella aertrycke cultures in 1:40 to 1:160 dilutions of serum. In one of Webster's experiments (21) in which 100 mice were artificially infected with B. pestis caviae seven of 26 survivors revealed agglutination with the homologous organism. Topley and Ayrton (22) and Topley, Ayrton, and Lewis (23) reported agglutinins in six of 68 in one series of induced infections and in seven of 55 in another. Hence the incidence of agglutinins was less than in the case of Amoss and greater than in that of the other investigators mentioned.

# Experiments on Pathogenicity

Subcutaneous and Intraperitoneal Inoculation.<sup>5</sup>—In determining the pathogenicity of the recovered organism a preliminary experiment demonstrated that, with a saline suspension of an 18 hour agar slant growth, 100 million organisms were uniformly fatal when injected intraperitoneally or subcutaneously into infant

<sup>&</sup>lt;sup>4</sup> We are indebted to Dr. L. T. Webster for several samples of normal mouse serum.

<sup>&</sup>lt;sup>5</sup> Ether anesthesia was used for all operative procedures.

mice, all of 24 dying within 48 hours. In titration of a similar preparation, 10,000 bacteria were found to kill, whereas 1,000 organisms produced a non-fatal enteritis. The induced experimental disease showed pathological changes indistinguishable from the natural affection. The organism was again recovered from the heart's blood, liver, and spleen of artificially infected mice in every instance.

Intraperitoneal administration of the same organism to ten recovered mice in the massive dose of 150 million organisms resulted in the death of only four, or 40 per cent. The disease was more protracted with death ensuing after as long as 15 days. Apparently a relative immunity to the microorganism had been acquired as result of a previous attack of the disease.

Ingestion of Organisms.—Characteristic experimental enteritis could be reproduced in infant mice by intraoral administration of cultures of the bacterium. Thus four of twelve animals receiving orally the cultures once on each of 3 consecutive days became affected.

Effects on Adult Mice.—Only relatively massive doses, that is, from 100 to 300 million organisms, induced fatal enteritis on intraperitoneal injection into adult mice. A smaller number of bacteria apparently was ineffective. It is probable that, inasmuch as the twenty mice employed for this test were obtained from the breeding room, they had been previously exposed to the infection. As already stated, recovery from the disease induces resistance to the effects of a later inoculation of the organism.

When the cultures were introduced intraorally by means of a syringe fitted with a blunt needle into four quarantined mothers, seven of twenty in the litters they were then suckling developed the non-lethal experimental disease. As a control, two mothers were treated similarly with paracolon bacilli with no evidence of illness in their litters; whereas two mothers given Salmonella aertrycke cultures per as showed in their litters of twelve young, characteristic mouse typhoid in all of them with a mortality of 80 per cent.

Possible Toxin Production.—Intraperitoneal inoculation of 0.5 cc. of a Seitz filtrate of a 48 hour broth culture resulted in non-lethal enteritis in two of seven infant mice. Since the filtrate was free from microorganisms there is a possibility that the bacterium produces a toxin, but this substance is either weak or small in amount.

The pathogenicity of the organism is of a lower grade than that of the enteritidis-aertrycke or mouse typhoid organisms (24, 25). This is substantiated by the apparent insusceptibility of adult mice, the lower mortality and morbidity among artificially infected young mice, and the low power of invasion in the natural disease. Infection with a mouse typhoid organism is followed by a septicemia that results in macroscopic changes in the liver and spleen with the infecting organism readily recoverable from these organs. In this epizootic it

was exceptional to recover the organism from the blood, liver, and spleen in the natural infection.

# **Carriers**

The production of the disease in infant mice following intraoral administration of the bacterial cultures to mothers led us to consider the possibility of conveyance of infection by way of the mother's milk. However, in six experiments we failed to recover the organism from the milk.

In the first three recovered mice which were cultured, the Salmonella organism was obtained in each instance from intestinal contents. Hence one may infer that carriers exist and that the source of infection is in fecal, rather than milk, contamination.

# DISCUSSION AND SUMMARY

It is now quite generally conceded that the presence in animals of bacteria of Salmonella type is indicative of an active, or potentially active, pathogenic agent (26). In the present instance bacteriological studies of the organs of acutely ill infant mice revealed the presence of one of the Salmonella group. It showed itself to be such by an absence of fermentation of lactose and saccharose, by other cultural reactions, and by cross-agglutination with certain members of the Salmonella group in very low dilutions of their antisera.6 However, the bacterium cannot be included in any of the known species of the group because of its indol-forming properties, differences in carbohydrate reactions, and specific serological reactions. Its presence in affected mice, growth on artificial media, ability to cause the disease following enteral and parenteral inoculations, and the fact that it can be recovered from artificially infected mice fulfill the postulates of Koch. Furthermore, agglutinins are present in the blood of recovered mice and not in that of normal animals, and recovery of mice

<sup>&</sup>lt;sup>6</sup> The organisms Salmonella enteritidis Gaertner, aertrycke (two strains), paratyphi, schottmilleri Seiffert (two strains), and suipestifer; and Eberthella typhi; Shigella dysenteriae Shiga, and paradysenteriae, variety Flexner, were used in the preparation of antisera. With the organism recovered in our studies cross-agglutination was present in titers not exceeding 1:200 only in the case of Salmonella enteritidis, aertrycke, schottmilleri Seifiert, and suipestifer antisera.

from natural infection evokes an apparent resistance against the special recovered Salmonella bacterium. The organism would appear to fall in the *Asiaticus* division of the genus, as designated by Castellani and Chalmers (27).

### CONCLUSION

A study is herewith reported of an epizootic limited in its occurrence to suckling and newly weaned mice. The primary causal agent appears to be a bacterium of Salmonella type.

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# THE METABOLISM OF COPPER AND IRON IN SPLENEC-TOMIZED RATS FREE FROM BARTONELLA MURIS INFECTION

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In previous studies it was found that the chemical changes in the blood of splenectomized rats suffering from Bartonella muris anemia consist of an increase in the total fats and fatty acids, a drop in lecithin and total cholesterol, a rise in blood chloride concentration, a moderate acidosis, and a variable drop in blood sugar. The liver glycogen is almost depleted. These changes are attributable either to the severity of the anemia or to the infection. Blood chemistry studies on splenectomized rats that were not carriers of Bartonella muris and in which no infection or anemia occurred revealed no significant deviation from the normal. Splenectomy in the rat free from Bartonella muris infection has no effect on metabolism that is demonstrable in the usual chemical analyses of the blood.

The present studies were undertaken in order to determine the effect of splenectomy on the metabolism of copper and iron in the rat free from latent *Bartonella muris* infection.

A relationship between the function of the spleen and the utilization of copper in the body was suggested by the experiments of Perla and Marmorston-Gottesman, and Perla, who observed the protective effect of an excess of copper in the diet on the natural resistance of albino rats to Bartonella muris anemia (1), Trypanosoma lewisi (2) and Trypanosoma equiperdum (3) infections. These diseases severely injure the spleen. In the case of Bartonella muris anemia a specific protective function of the spleen has been established (4). These studies suggested that the utilization of copper in the body may be dependent on splenic function. Further, it was found that in Trypanosoma equiperdum infection of albino rats there was a striking decrease in the concentration of copper in the spleen with no change in its con-

centration in the liver. The iron retention in the liver, however, was markedly increased (5).

The extensive literature on the relation of the spleen to the metabolism of iron has been critically reviewed in the monograph of Lauda and Haam (6), and by Wilson and Krumbhaar (7).

There is a considerable difference of opinion to be found in the literature concerning the effect of splenectomy on the concentration of iron in the liver, and on the metabolism of iron. Tedeschi (8) observed that the liver of the rabbit and of the guinea pig contains more iron pigment histologically after splenectomy than the organs of normal animals. Chevallier (9), however, found no increase in iron in the liver of rabbits during a period of 8 weeks following splenectomy. In a series of rabbits, chemical analyses of the iron content of the liver at intervals of 1 to 8 weeks after removal of the spleen did not demonstrate any increase in iron concentration in the liver according to Lauda (6). He observed, however, that in guinea pigs after a postoperative interval of 3 or 4 weeks the iron content of the liver seemed increased. Similar observations were made by Asher, Chevallier, and others. Lauda (6) believed that since the guinea pig was the only laboratory animal in which he found an increase of iron in the liver following splenectomy, some extraneous unknown factor was responsible for the increase, and that this did not reflect a direct disturbance in splenic function. Asher and Tominaga (10) found an increase in iron in the liver and kidneys of white rats following splenectomy, but this undoubtedly was associated with Bartonella muris anemia. Lauda observed no increase in the concentration of iron in the liver of splenectomized mice, nor in splenectomized doves.

In studies on rabbits, dogs, guinea pigs, and rats, Asher and his associates (11) found a marked and permanent increase in iron elimination after removal of the spleen. They believe that partial compensation for the loss of the splenic storehouse of iron was demonstrated by an increase in the iron content of the liver after splenectomy. In their opinion the spleen acts to prevent loss of iron from the body and therefore plays a principal rôle in the metabolism of iron.

Pearce and Krumbhaar and their associates (12) were unable to demonstrate a constant increase in the iron output following splenectomy in dogs. In those instances in which there was a significant increase in the elimination of iron in a splenectomized animal, it was associated with a marked secondary anemia. The exact reason for the anemia they were unable to determine. Wilson and Krumbhaar (7) estimated the iron balance in six dogs before and after splenectomy. In two intact dogs and in five dogs before and after control operative procedures other than splenectomy, the iron balance was found to be positive in the intact controls and in three of the operated controls, while a negative balance occurred in two of the operated controls which, however, had developed a mild postoperative anemia. Of the six splenectomized dogs, five showed an increased elimination of iron after splenectomy. This coincided with the period of developing

anemia. The authors suggested that, though the post-splenectomy anemia might have been the cause of the negative balance, splenectomy removed an iron depot and that the loss of iron thus produced was the cause of the anemia.

These investigators failed to stress the presence of latent Bartonella canis infection in the dog, a disease which often becomes manifest, following removal of the spleen, and is associated with a prolonged anemia (Kikuth (13)).

From a critical analysis of the literature Lauda concluded that there is insufficient proof that the spleen plays any rôle in the metabolism of iron or in hemoglobin formation.

# Methods

In our experiments, twelve male and female albino rats, free from Bartonella muris infection, were used. They were all 3 to 4 months of age and of approximately similar weights. The rats were kept in metabolism cages constructed with false bottoms so that the animals had no access to their feces. The cage rested on a funnel, the opening of which was covered with glass wool saturated with toluene and the stem of which fitted tightly into the neck of the collection bottle. Three rats were kept in a single cage. The diet consisted of purina in small checkers, and copper-free water which was supplied through drop bottles fitted with glass nozzles. Copper-free water was used throughout for washing everything that came in contact with the rats, as well as all the glassware used for the chemical determinations.

The urine and feces in each cage were collected twice a week. The urine was analyzed for uric acid (Benedict and Franke (14)), creatine and creatinine (Folin's microchemical modification (15)), total nitrogen (Kjeldahl), and copper (McFarlane (16)). The feces as well as the purina cubes were analyzed for total nitrogen (Kjeldahl), copper (McFarlane), and iron (modified Elvebjem and Hart method (17)). The elimination of iron in the urine is insignificant and was therefore disregarded.

The nitrogen metabolism and the metabolism of copper and iron were studied in the rats during a control period of 8 weeks prior to splenectomy and during 7 weeks after splenectomy. No differences in the results were noted in the males and females.

# Nitrogen Metabolism in Splenectomized Rats Free from Bartonella muris Infection

The splenectomized rats remained apparently well and showed no evidence of anemia. The nitrogen metabolism remained unchanged until 3 weeks after splenectomy. Following this there was an increased retention of total nitrogen with a decreased excretion of nitrogen in urine and feces. The creatinine and creatine remained strikingly constant throughout the experimental period. The uric acid remained practically unchanged (see Table I).

# Iron Metabolism in Splenectomized Albino Rats Free from Bartonella muris Infection

Following splenectomy there was an increased retention of iron during a period of 4 to 6 weeks after the operation. Then a drop

TABLE I

Nitrogen Metabolism in Normal and Splenectomized Rats Free from Bartonella
muris Infection

					D	aily ave	rage pe	r rat				
						Total	N			nine		
Date	Urine	Feces	Purina	Urine	Feces	Total excreted	Intake	Retention	Total creatinine	Preformed creatinine	Creatine	Uric acid
1933	cc.	gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Nov. 28-Dec. 4	8	1.8	9.6	207	125	332	394	62	9	5	5	1.20
Dec. 5-11	8.6	2	9.6	216	124	340	394	54	10	5	6	1.18
Dec. 12-18	11	1.9	9.6	206	121	327	394	67	11	5	7	1.31
Dec. 19-25	10	1.9	9.8	221	127	348	402	54	10	5	6	1.28
1933-34					1					1		[
Dec. 26-Jan. 1	10.5	2	9.6	215	125	340	394	54	11	6	6	1.30
Jan. 2–8	9	2	9.6	223	123	346	394	48	8	4	5	1.21
Jan. 9-15	9.5	2	9.7	203	126	329	398	69	10	6	5	1.3
Jan. 16-22	10.5		9.8	221	126	347	402	55	8	5	3	1.31
*Jan. 23–29	9	2	9.7	207	122	329	398	69	10	5	6	1.37
Jan. 30-Feb. 5	8.6	2	9.7	221	126	347	398	51	10	5	6	1.35
Feb. 6-12	11	1.9	9.8	209	124	333	402	69	10	5	6	1.34 1.30
Feb. 13-19	8	2	9.9	201	105	306	406	100	9	4	6	1.34
Feb. 20–26	7	1.9	9.8	185	108	293	402	109	11	5	7	1.36
Feb. 27-Mar. 5	8	1.9	9.8	187	105	292	402	110	10	5 5	6	1.32
Mar. 6–12	7	2	9.9	188	106	294	406	112	10	3	0	

<sup>\*</sup> Jan. 23, splenectomized.

occurred and the iron retention decreased to the level observed before splenectomy. This was unassociated with any evidence of hemoglobin destruction.

# Copper Metabolism in Splenectomized Rats Free from Bartonella muris Infection

From Table II it is evident that a disturbance in the copper metabolism occurred, following removal of the spleen. For a period of 2 weeks following splenectomy the copper balance remained positive

TABLE II

Copper and Iron Metabolism in Normal and Splenectomized Rats Free from

Bartonella muris Infection

	Daily average per rat													
Date			Cu				Fe							
	Urine	Feces	Total excreted	Intake	Retention	Feces	Intake	Reten- tion						
1933	mg.	mţ.	mg.	mg.	mg.	mţ.	mg.	mg.						
Nov. 28-Dec. 4	0.0052	0.0980	0.1032	0.1383	0.0351	1.976	1.805	-0.171						
Dec. 5-11	0.0062	0.1086	0.1148	0.1383	0.0235	1.661	1.824	+0.163						
Dec. 12-18	0 0052	0.1061	0.1113	0.1383	0.0270	1.789	1.843	+0.054						
Dec. 19-25	0.0054	0.1055	0.1109	0.1411	0.0302	1.705	1.882	+0.177						
1933-34														
Dec. 26-Jan. 1	0.0070	0.1090	0.1160	0.1383	0.0220	1.802	1.843	+0.041						
1934		ł			ĺ			,						
Jan. 2-8	0.0063	0.1115	0.1178	0.1383	0.0205	1.835	1.800	-0.035						
Jan. 9-15	0 0063	0.1099	0.1162	0.1397	0.0235	1.663	1.755	+0.093						
Jan. 16-22	0 0067	0 0978	0.1045	0.1411	0.0366	1.674	1.774	+0.100						
	<del>\</del>		·		\		ļ							
*Jan. 23-29	0.0066	0.1110	0.1176	0.1397	0.0221	1.437	1.756	+0.319						
Jan. 30-Feb. 5	0 0074	0.0965	0.1039	0.1397	0.0358	1.347	1.756	+0.409						
Feb. 6-12	0 0057	0 1437	0.1494	0.1411	-0.0083	1.613	1.774	+0.161						
Feb. 13-19	0 0065	0 1440	) 0.1505	0.1425	(-0.0080	1.382	1.792	+0.410						
Feb. 20-26	0.0058		0.1391	0.1411	+0.0020	1.650	1.774	+0.114						
Feb. 27-Mar. 5	0 0051			0.1411	-0 0000	1.610	1.774	+0.164						
Mar. 6-12	0 0030	0, 1413	3 0 1463	0.1423	-0.0038	1.734	1.792	+0.058						

<sup>\*</sup> Jan. 23, splenectomized.

and the retention was the same as in the normal rats during the preoperative period. Then excretion of copper in the feces increased and a negative copper balance resulted which persisted during the remainder of the experimental period. No change in the elimination of copper in the urine was noted.

# DISCUSSION

In previous experiments (18) the changes in the concentration of certain chemical constituents of the blood in splenectomized rats of Bartonella carrier stock were found to be associated with Bartonella muris infection and anemia and were not due to the removal of the spleen itself. In rats free from this infection no change from the normal concentration in the blood constituents occurred. Removal of the spleen in albino rats free from latent Bartonella muris infection and in which no anemia develops, results in a disturbance of the copper metabolism which becomes manifest 2 weeks after operation. The increased elimination of copper in the feces is associated with a transiently increased retention of iron, but the negative copper balance persists. No disturbance in nitrogen metabolism occurs.

The reciprocal relationship in the metabolism of copper and iron is difficult of explanation, but bears some resemblance to the relation of calcium and phosphorus metabolism.

The variability in results of experiments on the metabolism of iron in splenectomized animals, found by previous investigators, was doubtless due in part to the presence of latent infections that became manifest following the removal of the spleen. The complicating anemia that was observed in dogs and rats was due to infection and not to an interference with hemoglobin formation resulting from removal of the spleen. (For a discussion of the spleen and latent infection, see the monograph of Perla and Marmorston.1) Many species of dogs are carriers of Bartonella canis (Kikuth (13), Perard (19), Regendanz (20)), and under certain conditions an anemia develops following splenectomy due to a flare-up of the Bartonella canis infection. Most species of rats are carriers of Bartonella muris, and splenectomy is followed by an active infection associated with a fatal anemia. Mice are carriers of eperythrozoon coccoides and Bartonella muris infections that become active following splenectomy (Schilling (21), Eliot and Ford (22), and Marmorston (23)). Marmorston observed that a third latent infection in the mouse, Klossiella muris of the kidney, was unfavorably influenced by splenectomy. Splenectomy in cattle, horses, and sheep results in activation of latent piro-

<sup>&</sup>lt;sup>1</sup> Perla, D., and Marmorston, J., Relation of the spleen to resistance, to be published.

plasmidae diseases often associated with severe anemia and death (24). Further studies on the effects of splenectomy on metabolism can be reliable only if the presence of latent infections has been rigidly excluded.

From our experiments, therefore, it seems justifiable to conclude that the disturbance in the copper and iron metabolism noted in the animals following splenectomy is due to the removal of the cells of this organ and not to any known extraneous factor.

No disturbance in copper metabolism was noted for a period of 2 weeks after removal of the spleen. It is possible that some substance elaborated by the spleen and essential for the utilization of copper is stored elsewhere in the body and that this supply is exhausted after a period of 2 weeks.

### SUMMARY AND CONCLUSIONS

Removal of the spleen in albino rats free from *Bartonella muris* infection is followed by an increased elimination of copper in the feces, which commences 2 weeks after splenectomy. This is associated with a persistent negative copper balance.

An increased retention of iron occurs during a period of 4 to 6 weeks after splenectomy with a return of the iron metabolism to normal after this period.

No disturbance in creatine or creatinine metabolism occurs. The uric acid amount is unchanged. There is an increase in the retention of nitrogen, which is first noted 3 weeks after splenectomy.

The spleen is essential for the utilization of copper in the body.

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# STUDIES ON WHOOPING COUGH\*

# I. Type-Specific (S) and Dissociation (R) Forms of Hemophilus

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(Received for publication, July 5, 1934) In the recent revival of interest regarding the etiology of whooping cough in America, two outstanding beliefs are: (a) that the disease is caused by H. perlussis (the bacillus of Bordet and Gengou), or (b) that the specific agent is a filtrable virus. Of the two opinions, the first named is far more generally accepted and stands practically unquestioned in Europe (1). A third hypothesis, based upon analogy with Shope's recent work on swine influenza (2), which combines the two, proposes the possibility that a filtrable agent and H. pertussis act together to produce the disease (3). Of the three theories the first named has seemed to us most acceptable (4), and for this reason we have undertaken and are herewith reporting detailed studies of the biological character of H. perlussis, with a view to clarification of some of the confusion existing in regard to its phases and dissociation forms and with the definite object of bringing these into conformity with the terminology currently employed in the description of bacteria and their variants.

II. perlussis when first isolated differs from forms found in old laboratory cultures. This difference was first described by Bordet and Sleeswyk (5) who noted that the freshly isolated organism differs serologically from old stock strains. Bordet believed that the difference was due to adaptation of the latter to agar media. Krumwiede, Mishulow, and Oldenbusch (6) described two agglutinative varieties

<sup>\*</sup> This study was made possible by a grant from the Rockefeller Foundation.

or types, A and B; they failed to confirm Bordet's conclusions referred to above. From inspection of the data presented by these workers, it seems probable that their two types represent subvarieties of old stock cultures. Leslie and Gardner (7), who have recently restudied the problem, conclude that *H. pertussis* is a uniform species without fixed types when first isolated, but after subculture upon various laboratory media, it passes through a series of antigenic stages which they have called Phases I, II, III, and IV. Lawson (8) has been able to confirm their work in general but was never able to obtain their Phase II. It is not impossible that this phase exists, although we have never noted it, and that it may represent an intermediate transition form between freshly isolated strains and older stock variants.

From the findings to be reported below, it seems certain, as first suggested by Leslie and Gardner, that recently isolated strains (Phase I) correspond to the virulent or S forms and that the antigenically different stock strains (Phases III and IV) are the same as the avirulent or R variants of other bacteria. Hereafter, therefore, both for purposes of convenience and in conformity with current nomenclature, the two forms will be referred to as S and R forms. It should follow from this that the freshly isolated form of H. pertussis (S) represents the pathogenic phase. On this assumption transmission experiments have been commenced in an effort to produce whooping cough in apes with the S form. To eliminate the possibility of there being a virus component in the inoculum, we have maintained the organism in the S phase, by methods to be referred to below, for several months before attempting transmission experiments.1 While so engaged, we have undertaken detailed studies of the S phase in order to establish unmistakable criteria for its identification and for its differentiation from R forms. For this purpose observations have been made upon the cultural, morphological, toxic, serological, electrophoretic, and isoelectric properties of all strains studied. It is with a consideration of these findings that the present communication is chiefly concerned.

<sup>&</sup>lt;sup>1</sup> In a preliminary communication (4) we have reported what we believe to be the successful transmission of whooping cough by means of this theoretically virus-free pathogenic S form and unsuccessful transmission with the R form in chimpanzees. The S form had been subcultured more than 60 times over a period of 8 months.

## Methods

Materials.—We have studied 98 strains of H. pertussis. Fifty-nine were freshly isolated strains and were obtained from the following sources: Cleveland strains, 12 from Dr. Toomey of the Cleveland City Hospital and 30, isolated by us, mainly from cases of whooping cough in Dr. Gerstenberger's Pediatric Clinic of the Lakeside Hospital Out-Patient Department; Boston strains, 4, from Dr. G. M. Lawson and Dr. Hans Zinsser of The Harvard Medical School; Chicago strains, 3, from Dr. L. W. Sauer of the Evanston Hospital; Albany strains, 3, from Dr. A. B. Wadsworth of the New York State Board of Health; California strains, 3, from Dr. A. P. Krueger of the University of California; and Danish strains, 3, sent us by Dr. J. J. Miller who at the time was associated with the Statens Serum Institute in Copenhagen. Thirty-nine strains were laboratory stock cultures. Nineteen of these of widely scattered origin, including an original Bordet strain, were kindly sent to us by Dr. Toomey. The remaining 20 came from: New York, 2 (Dr. Park); Albany, 3 (Dr. Wadsworth); Boston, 2 (Dr. Lawson); and 13 were derived from our own freshly isolated strains.

Cultural Methods.—For the isolation of H. pertussis from active cases of whooping cough, the cough plate method of Chievitz and Meyer (9) was used.<sup>2</sup> For cough plates and for cultivating freshly isolated strains, the Bordet-Gengou potato-glycerin-blood agar medium used was essentially that recommended by Lawson and Mueller (10) except that sterilization was done by autoclave with 15 pounds of pressure at 121°C. for 20 minutes instead of by the Arnold sterilizer. Defibrinated or citrated human blood, either placental or obtained by venepuncture, was used in 25 to 35 per cent concentration. Plates were always poured freshly with blood usually less than 5 days old and never more than 1 week old. Plates, slants, and Blake bottles were poured thick. Old stock cultures were grown on chocolate blood (5 to 10 per cent), glycerin, agar (1.5 per cent) medium.

Merphological Studies.—Colonies were examined under the Zeiss colony microscope with both oblique and indirect illumination using magnification × 20. Smears were studied with Gram stains. For capsular studies, Wright's stain diluted 2:1 with glycerin, as recommended by Lawson (8) was used.

Determination of Toxicity.—This was determined on rabbits by intradermal injection using Berkefeld filtrates of the organisms and on guinea pigs by the intraperitoneal method used by Leslie and Gardner (7).

Methods of Agglutination.—Agglutinating sera were prepared by intravenous injection of rabbits with washed organisms resuspended in formalized (0.2 per cent) normal salt solution. Titers ranged from 1:2,000 to 1:20,000.

Forty-two attempts in all were made to recover the organism. The day of the disease ranged from the 2nd to the 28th. In cases seen during the 1st week the number of positive plates was 100 per cent (6 cases), in the 2nd week 100 per cent (10 cases), in the 3rd week 59 per cent (22 cases), and in the 4th week 50 per cent (4 cases), an average of 76 per cent for all cases.

All agglutination tests were done macroscopically. Organisms were washed twice with distilled water and resuspended in formalized saline in a concentration of 2 billion per cc. before addition to serum dilutions. When cultures were granular, glycocoll-sodium phosphate-sodium acetate buffer solutions (11) at pH 7.0 were substituted for the saline (12). Readings were made after 18 hours incubation at 37°C. Incubation at 56°C. occasionally resulted in the appearance of an inhibition zone (13) and was discontinued accordingly.

Absorption of Agglutinin.—Organisms were treated as for agglutination. The absorbing dose was the growth obtained from 6 to 8 Blake bottles. Serum dilutions used ranged from 1:10 to 1:100 depending upon the agglutinative titer of the serum tested. Serum-bacteria mixtures were incubated for 3 hours at 37°C. and were placed in the ice box overnight. Reciprocal absorption as recommended by Krumwiede, Cooper, and Provost (14) was carried out in all cases.

Measurement of Potential.—Cataphoretic migration velocities were determined by means of the Northrop-Kunitz microcataphoretic apparatus (15) and are reported in terms of microns/second/volt/centimeter. Before making readings, the bacteria were washed 3 times with distilled water and were then suspended in glycocoll-sodium phosphate-sodium acetate buffer at pH 7.0, molar concentration 1:100. For determination of isoelectric points, the same buffer solution at varying pH's in molar concentration of 1:25 was used.

### RESULTS

Before proceeding to a detailed consideration of our experimental findings, a brief account of the transition from the S forms of H. pertussis to the R dissociants is in order. When first isolated upon Bordet-Gengou medium cough plates, we have found the organism to be a small Gram-negative coccobacillus which agglutinates to full titer with sera prepared with freshly isolated, or S strains, agglutinates in very low dilution or not at all with anti-R sera and is incapable of growth upon chocolate agar. After continued subculture upon Bordet-Gengou medium, it begins first to grow scantily and later more luxuriantly upon the coagulated blood medium. When this stage is reached, the organism more than doubles its cataphoretic migration velocity and will agglutinate with R sera. While in transition, cataphoretic studies show organisms moving at both the slow rate of S and the more rapid rate of R forms. The latter probably represent the developing R variants. Eventually, as growth becomes more luxuriant on the chocolate agar, the S form drops out and the fully developed R form is established. When this is accomplished the organism is no longer ovoid but is a long, thin bacillus and no longer

agglutinates with S sera. With this brief summary, we may pass on to a detailed consideration of the recently isolated S form and to a discussion of its essential differences from R forms.

Cultural Differences between S and R Strains.—When a positive cough plate from a case of whooping cough is examined under the colony scope at the end of 24 hours, typical colonies of H. pertussis appear as minute, shiny, greyish white, opaque, dome-shaped or pyramidal pin-points. They are quite distinctive and are readily differentiated from the colonies of other organisms, including H. influensae (16), appearing upon the plate. After 48 to 72 hours incubation. they are fully developed and take on the character of a domed, glistening half-pearl, measuring 1 to 2 mm. in diameter. The edge is quite smooth, although occasionally if the medium is dried out, there may be fine stippling. Larger colonies, due to piled up growth, may take on a moist, glossy, pearly white appearance. By 48 to 72 hours most colonies will be surrounded by a small though definite zone of hemolysis. On subculture, if growth is heavily streaked, the hemolysis is much more conspicuous. R forms on Bordet or chocolate agar media grow more rapidly and present a flatter, less compact appearance. Their color is a dull or dirty grey rather than pearly grey. They are not glossy. Although their outline is not grossly rough as is the case for example, with R pneumococci, it lacks the sharp definiteness of contour of S forms. The zone of hemolysis so typical of S strains is not present around R forms.

A characteristic difference between S and R forms is found in their behavior in saline suspension. S strains are always readily emulsified when taken off solid media and are stable. R forms at times, however, emulsify with difficulty and not infrequently are granular; on occasion, they may be so unstable in suspension as to make agglutination tests, even in buffer solution, practically impossible. This instability in suspension is frequently a characteristic of the R forms (c.g. R pneumococci) of other bacteria.

Morphological Differences between S and R Strains.—The recently isolated or S form when stained by the method of Gram is characteristically a small Gram-negative coccoid or oval bacillus. The organisms are quite uniform in appearance and average about  $1.0 \times 0.5$  micra in size. Paired organisms are not infrequent. Longer bacillary

forms are rare (Fig. 1). The R form is typically a much longer bacillus, running as high as 4 or 5 micra in length but retaining about the same diameter as the S form. Occasionally ovoid bacilli may be seen (Fig. 2) in films of R strains. Attempts to show a capsule with the usual staining methods have been unsuccessful in our hands and in those of Lawson, but when Wright's stain diluted 2:1 with glycerin as suggested by Lawson is used, definite capsules as described by him (8) may be made out in young cultures of S forms (Fig. 3), while indefinitely outlined or doubtful capsules appear in R strains.

Toxicity.—At the time that Bordet and Sleeswyk noted the antigenic differences between recently isolated and stock strains of H. pertussis, they demonstrated that the former, upon intraperitoneal inoculation of guinea pigs, were toxic, whereas the latter were not. Leslie and Gardner (7) have repeated this work and state that their Phases I and II were toxic for guinea pigs and III and IV were not. Bordet's finding that there is no multiplication of bacteria within the animal's peritoneal cavity and his view that death is purely toxic in its mechanism received support from these authors. Lawson (8), elaborating upon the work of Teissier, Reilly, Rivalier, and Cambassedes (17) has shown that there is a toxic filtrate (endotoxin) obtainable from Phase I (S) strains which produces a skin reaction in guinea pigs and which is neutralizable both in vivo and in vitro by immune serum. He was unable to show similar toxic properties in Phase III and IV (R) cultures.

We have made no effort to study this aspect of the problem in detail. However, in seeking criteria for the identification of S strains, we have tested a few of our cultures when indicated by intradermal injection of rabbits with filtrates and by intraperitoneal injection of guinea pigs according to the technic of Leslie and Gardner and have consistently found our S strains to be toxic and our R strains to be non-toxic.

Serological Differences between S and R Strains: (a) Agglutination.— Leslie and Gardner (7) working with 16 strains isolated by them and with 4 additional fresh strains obtained from Denmark and with 12 stock cultures derived from the Lister Institute and from New York, concluded that their freshly isolated strains were a uniform antigenic species without fixed varieties or types (Phase I) and that after isolation and repeated laboratory subculturing on various media, they passed over through a transitional phase (II) into Phases III and IV, the phases represented by their stock cultures. Lawson (8) working with Leslie's phase sera studied 18 freshly isolated strains in Boston and found that they were Phase I (Leslie and Gardner). Certain stock strains in his possession were Phases III and IV. In an effort to extend this work and particularly with a view to amplifying it in the direction of discovering whether S forms (Phase I) were a uniform type wherever derived, we have studied 59

TABLE I

Summary of Agglutination Tests of S and R Strains of H. pertussis with S and R Sera

+ indicates agglutination to full titer of the serum employed. - indicates no agglutination or slight agglutination only. Nine different S and 7 different R sera were used in the tests.

	S se	rum	R serum			
Strains of H. periussis	No. of strains	Results	No. of strains	Results		
S strains						
Cleveland	39	1 +	24			
Boston	4	+	4	_		
Chicago	3	+	3	-		
California	3	+	1	-		
Denmark	3	+	2 .	-		
R strains						
Cleveland (Toomey)	18	_	18	+		
Cleveland (from S strains)		_	13	;		
Boston		-	2	+		
New York City	2	-	2	1		
New York State		_	3	1		

freshly isolated strains obtained from widely distributed sources. They include strains from Boston shown by Dr. Lawson to be Phase I with English phase serum, strains from Denmark, and strains obtained in this country from Boston, Albany, Chicago, San Francisco, and Cleveland.

All of these strains, with the exception of the Albany ones, which were somewhat older and presumably had dissociated to the R form, have agalutinated to full titer with serum made with strains freshly

isolated by us or with sera made with S strains selected from each of the sources noted above. All of them failed to agglutinate or agglutinated only in low titer with sera made with stock (R) strains. These findings are summarized in Table I. In brief, all recently isolated S strains of H. pertussis tested by us, although obtained from widely distributed sources, are identical by the method of agglutination and are agglutinatively distinct from R forms.

We have not made detailed efforts as yet to differentiate subvarieties of R strains (Phases III and IV). As stated above, we have not noted Leslie and Gardner's Phase II (transitional S strain). Every one of the R strains including a "Phase III" sent us by Dr. Lawson has agglutinated to full titer with serum made with Strain A (Mishu-

TABLE II

Reciprocal Absorption Tests with Scrum and Organisms of S Strains of H. pertussis from Cleveland (H16) and Boston (Z)

	Organism agglutinated	Titer before		er after absorption ith		
			Strain H16	Strain Z		
Serum H16	H16	1:2,560	>1:100	>1:100		
	Z	1:2,560	>1:100	>1:100		
Serum Z	H16	1:20,480	>1:100	>1:100		
	Z	1:20,480	>1:100	>1:100		

low) which was designated Phase IV by Leslie and Gardner (7), and Lawson (8). This has been true of all stock strains obtained from outside sources as well as all R forms derived from our own S strains. One strain (M1, obtained from Dr. Park) has puzzled us, as although in all other respects it has behaved as an R variant, its serum has occasionally agglutinated our S strains even though it has not been agglutinated by the anti-S sera. We are planning to study this strain and other aspects of R dissociants in the near future.

(b) Agglutinin Absorption.—In order to establish the fact that all our widely derived S strains, seemingly the same by agglutinative methods, are serologically identical, we have tested them further by absorption of agglutinin. In all cases we have used the method of reciprocal absorption insisted upon by Krumwiede (14). We have not tested all

strains but have selected single samples from each of the sources noted above. By this method in all cases tested, it has been found that absorption is reciprocally complete. Table II shows a typical experiment using S strains from Cleveland and from Boston.

As noted above, studies concerned with the classification of subvarieties of R forms have not yet been undertaken in detail. However, such work as we have done by the methods of agglutination or agglutinin absorption, have not indicated that mutually exclusive, sharply defined phases are the rule.

Cataphoretic Differentiation of S and R Strains.—Early in our studies of H. pertussis we noted and reported the fact (18) that recently isolated (S) strains could be sharply differentiated from stock (R) forms by means of their cataphoretic migration velocities in an electropho-

TABLE III

Summary of Readings Made of Cataphoretic Velocities of S and R Strains of II. pertussis

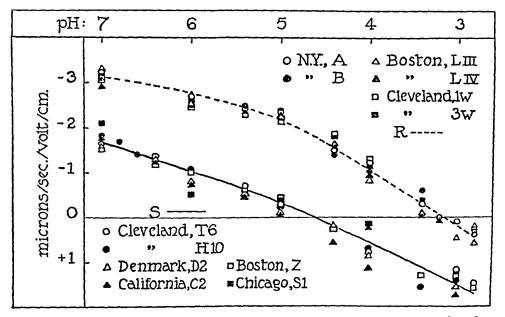
Organisms washed 3 times with distilled water and resuspended in  $\mu/100$  glycocoll-sodium phosphate-sodium acetate buffer, pH 7.0.

	No. of strains	No. of determinations	Average  µ/sec./v./cm.
S strains	53	201	1.78
R strains	39	162	4.20

retic cell. At that time we had worked with 16 S and 6 R strains. Since then we have extended our observations to include 53 S and 39 R cultures and have consistently confirmed our original findings. All S strains have shown slower migration velocities than R variants. The velocities for S strains under standard conditions have averaged  $1.76 \,\mu$ /sec. / v. / cm. (range 1.2 to 2.3) and for R strains the average reading has been  $4.2 \,\mu$ /sec. / v. / cm. (range 3.9 to 4.6). A summary of the findings appears in Table III. These results show clearly the striking difference existing between the migration rates of the two forms, the R form moving at a rate over twice that of the S form. Although the individual variations in the two categories are fairly wide, the great majority of readings have fallen fairly close to the mean, and further, these occasional variations have been noted in

readings made at different times upon a single strain. Some of these have been accounted for on the basis of technical difficulties.

Differences between toxigenic and non-toxigenic (S and R) diphtheria bacilli and between S and R pneumococci have already been noted (19). The findings herewith reported for *H. pertussis* would seem to parallel these and constitute a further basis for considering recently isolated (S) strains, virulent forms, and stock strains, avirulent or R dissociants.



Text-Fig. 1. Curves showing (a) the effect of varying pH upon migration velocities of S and R forms of H. pertussis and (b) their isoelectric points. The curve for the S forms is the solid line and for the R forms, the broken line. Readings for individual strains are indicated by the symbols shown on the chart.

Certain observations have been made in regard to the relationship existing between migration velocities and variation from S to R and back again. When an S strain is maintained upon Bordet-Gengou medium containing a high percentage of blood (25 to 35 per cent), it retains its typical slow rate for a long period of time, perhaps indefinitely. When the proportion of blood is reduced to 5 to 10 per cent, faster moving organisms begin to appear among the slower ones, the ratio being about 2:1. Finally, when the strain has adjusted to

chocolate agar, the slowly moving organisms are completely replaced by those of higher migration velocities and the strain is now completely R in all respects. If now the R strain is grown again on Bordet-Gengou medium with high blood percentage, provided it has not been R too long, it will revert to its slower rate and will assume all the characteristics of S strains.

One of our R strains, M1, has regularly shown a migration velocity (3.1  $\mu$ /sec./v./cm.) intermediate between those characteristic of S (1.76) and of R (4.2) forms. It is interesting to note that this is the strain referred to above which has behaved in inconsistent fashion agglutinatively and which is being investigated further in our projected detailed studies of R variants.

The Isoelectric Point of S and R Strains.—The finding of these characteristically different cataphoretic velocities for S and R strains suggested the advisability of an investigation of their respective isoelectric points. Fifteen determinations upon 12 representative S strains and 34 determinations upon 24 R variants have been made. The results show that S and R forms may be sharply distinguished by their respective isoelectric points. For recently isolated strains (S), the isoelectric point falls between pH 4.4 and 4.8, average, pH 4.61. In the case of R forms, the isoelectric point falls between pH 2.8 and 3.6, average, pH 3.19. Text-fig. 1 shows the curve for 6 representative strains in each of the categories. Differences in the isoelectric points of a virulent strain of the bacillus of rabbit septicemia and its less virulent dissociant have been reported by De Kruif (20). The finding of these differences in their isoelectric points for S and R strains of H. pertussis. constitutes further evidence in favor of their representing respectively the pathogenic form of the organism and the avirulent variant.

The Maintenance of S Forms.—Freshly isolated strains of H. pertussis persist, perhaps indefinitely, in the S form, as determined by the criteria enumerated in the foregoing sections, provided that they are kept growing upon Bordet-Gengou potato-glycerin-blood agar containing no less than 25 per cent of freshly defibrinated blood. We have 6 strains (H8, H10, H12, H15, H16, and H19) that have retained their cultural, hemolytic, morphological, serological, and cataphoretic S characteristics, under these conditions of cultivation, for periods ranging from 21 to 31 months. Strain Z used for the successful ape trans-

mission experiment referred to above, was over 8 months old and had been subcultured 60 times when used for inoculation.

If the proportion of blood is allowed to fall below 20 per cent, dissociation of S to R forms occurs. If the cultures are now seeded upon

TABLE IV

Criteria for Identification of S Form of H. pertussis and for Its Differentiation from R Forms

	S forms	R forms
Relationship to whooping cough	May be recovered in first 2 wks. of disease if suitable measures are used in practically all cases	Not present in first 4 wks. of disease
Cultural charac- teristics	Dome-shaped, half-pearl. Sharply circular outline	Outline circular but not sharply circumscribed. Elevation rather flat and not compact
	Glistening, mucoid or moist appearance	Not glistening in appearance
	Pearly greyish white color	Dull, dirty light grey color
	Surrounding zone of hemolysis	No hemolysis
Stability of suspen- sion in 0.85 per cent NaCl	Emulsify smoothly to form stable suspension	Emulsify with difficulty. Frequent instability with autoagglutination
Morphological	Small Gram-negative coccobacil- lus, 1.0 x 0.5 $\mu$	Long, thin bacillus, $4.5 \times 0.5 \mu$
	Generally uniform in size	Considerable variation in size
	Capsule present	Capsule probably not present
Serological	Agglutinate to full titer with sera made with recently isolated (S) strains, wherever derived Complete reciprocal absorption	
	with other S strains	
	No absorption of agglutinins from R sera	No absorption of agglutinins from S sera
Toxicity	Toxic for guinea pigs upon intra- peritoneal injection	Not toxic
Cataphoretic	Under standard conditions migration velocity is 1.75 $\mu$ /sec./v./cm.	v./cm.
	Isoelectric point: pH 4.6	Isoelectric point: pH 3.2

chocolate agar, they will grow luxuriantly in the R form. Strains thoroughly adapted to the last named medium will grow upon brain agar medium. R strains which have been growing for periods of

designated the S form and the laboratory variants the R form of *H. pertussis*. Whether the R variants fall into sharply defined phases as pointed out by Leslie and Gardner, remains to be confirmed. Lawson and the writer have never noted their "Phase II" and Toomey (21) is in agreement with us that sharply defined, mutually exclusive subvarieties of R variants probably do not exist. We are undertaking further studies of this somewhat mooted point.

Dawson, as a result of detailed studies concerned with the dissociation of pneumococci (22) and of streptococci (23) has shown that these organisms have three variant forms and he proposes that the terminology currently employed for pneumococcal variants be changed to conform with the terms used in the description of corresponding variants of other bacterial species. The first form, encapsulated, at present called S, he designates mucoid (M); the second, at present R, he calls smooth (S); and the third, a new and distinct, grossly rough variant which he describes for the first time, he would call rough (R). Hadley (24) has found that most organisms show these three chief colony forms. From conversation with Dr. Dawson, it seemed that our S which is encapsulated and has a moist mucoid colony has the character of his M form. Whether the subvarieties of R variants (III and IV of Leslie and Gardner, A and B of Mishulow) correspond to his S and R, remains to be seen. For the time being it has seemed more practical to use the terms S and R as generally employed. As we study H. pertussis variants further, it is possible that these three chief colony forms, constituting the usual pattern for other bacterial species, may emerge as well defined types.

The finding that *H. pertussis* when first isolated is a single specific serological type, in S form, and that this antigenic phase may be maintained by suitable cultural management has certain definite implications. One bears upon further transmission experiments directed toward the establishing of the etiological relationship of the organism to whooping cough. Working with monkeys, Sauer (25) produced suggestive manifestations of the disease in 8 out of 76 attempts. It is very probable that his failures may be laid to the use of R forms as inoculum. Criticism directed against the conclusiveness of the occasional successful transmission experiment (Sauer (25), Rich et al. (26), MacDonald and MacDonald (27)) with freshly isolated *H. pertussis* put forward by those entertaining the combined *H. pertussis* 

reported upon (4) and still being carried out in this laboratory, by sub-417 culturing the theoretically pathogenic S form long enough to preclude

A second implication of importance related to the preparation of H. pertussis vaccines. Madsen (28), Sauer (29), and Frawley (30), the last worker using Krueger's specially prepared H. pertussis vaccine (31), have recently reported favorably regarding protection against whooping cough by means of suitably prepared vaccines. Sauer following the lead of Madsen insists upon the use of freshly isolated organisms; Krueger's special vaccine is made from similar strains. ready identification of the S form of H. pertussis and the practicability of its maintenance brought out above puts the preparation of antigenically effective vaccines upon a sound basis. In cooperation with Dr. J. A. Doull of the Department of Hygiene and Bacteriology and Dr. H. J. Gerstenberger of the Department of Pediatrics, we have begun a carefully controlled study of the prophylactic value of H. pertussis vaccines made from organisms shown to be in the S form according to the criteria outlined above. By the same token, the therapeutic value of H. pertussis vaccine in active cases of the disease can be determined only after carefully controlled studies have been carried out with such antigenically effective preparations.

# CONCLUSIONS

- 1. Fifty-nine recently isolated and 39 laboratory stock strains of II. pertussis have been studied with respect to their cultural, morphological, toxic, serological, and cataphoretic properties.
- 2. Recently isolated strains, whether derived from cases of whooping cough in Europe or in this country are a single specific serological type which should be designated the S form of H. pertussis. 3. Criteria for the identification of the S form are given.
- 4. The S form may be maintained in this stage by suitable cultural methods. If grown upon media deficient in blood, it will dissociate into an antigenically different form which should be designated the R form in keeping with current terminology employed in describing

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# EXPLANATION OF PLATE 18

- Fig. 1. S form of H. pertussis showing coccobacillary forms. Occasional pairing of organisms appears.  $\times$  1,250.
  - Fig. 2. R form of H. pertussis showing long bacillary morphology. × 1,250.
- Fig. 3. Capsules of S forms of H. pertussis. Wright's stain diluted 2:1 with glycerin was used. The capsules appear bluish pink in the original smears. The color did not photograph well.  $\times$  1,250.



# A COMPARISON OF OCULAR MICROMETRIC AND PROJECTOSCOPIC METHODS OF ESTIMATING GROWTHS IN TISSUE CULTURES

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(Received for publication, July 2, 1934)

An important part of investigations dealing with tissue cultures is the estimation of the amount of growth occurring under a given set of conditions; and for this purpose the procedure described by Ebeling (1) has proven the most satisfactory. Those employing this method recognize that it does not give an accurate picture of the total number of cells that have grown in the culture, because the areas drawn represent extension in only two dimensions, and fail to consider the third as represented by the thickness of the growth. The critics of this procedure, nevertheless, have offered no practical substitute; and many investigations continue to be recorded in which no quantitative criteria are applied. The microscopic appearance of the cells is obviously important in helping one to form a final judgment, and should not be neglected. If, therefore, the data for both quantitative and qualitative criteria could be gathered from a single manipulation, certain distinct advantages would follow. The purpose of this communication is to describe a simple method whereby fairly satisfactory quantitative data can be easily acquired.

The method of Ebeling necessitates considerable apparatus. For the drawing of the silhouette some form of projectoscope is required, and if the source of light is very hot the culture must be protected by a heat-absorbing chamber. Unless the apparatus is in a warm room the culture flasks or slides are subjected to cooling with a consequent retarding of growth. The accuracy of the results depends, naturally, upon the experience of the observer in outlining the silhouettes and in planimetry, and also upon an understanding of the relationships of these two mancuvers one to the other. In fact, the limitations of

tissue culture technique make too minute measurements unnecessary. A planimeter is, however, needed for the determination of the area occupied by the drawn silhouette. As eight or more growths are generally used in each portion of an experiment, the time required for making tracings and measurements is no small item; and this must be followed by calculation of increments of growth and of comparative indices. Usually the increment represents the average of calculations from a number of growths, and the indices are quotients of the average growth under some special experimentally arranged condition divided by an average growth under normally arranged condition.

After having become familiar with the above described technique, it seemed to us desirable to determine whether a simpler method might not give almost as satisfactory results, and thus be applicable in laboratories where the more elaborate apparatus is not available.

# Methods

Transplants of actively growing fibroblasts were divided into an equal number of pieces, half of which were placed in Carrel flasks containing media representing an abnormal environment and half in a normal environment. The transplants were identified so that their origin could be traced. Their increment of growth was then estimated by one of us with the projectoscope, planimeter, adding machine and slide rule, according to Ebeling's technique. Simultaneously another observer estimated the increments of growth as follows: An eyepiece disc micrometer was placed on the diaphragm of a 10 × ocular which was used with an 180 mm, objective for the measurements of the transplants. The micrometer scale contained 100 equal divisions, with every tenth division numbered; and measurements were carried out to the nearest unit of 5. As the transplants originally were irregularly rectangular, the first measurement was made with the micrometer scale extending through the longest axis of the transplant; the ocular was then turned 90° and a second measurement was made through the other axis. If the transplant was very irregular in outline allowance was made so that approximately equal portions would extend outside and inside an imaginary line projected at right angles to the end of the axis being measured. On subsequent days as the growths assumed more and more a circular outline, the two axes were measured in the same manner. At the same time note was made of the qualitative character of the growth and the various elements composing it, and of the thickness of the culture by focusing at different levels.

The calculations of increments were made on the assumption that the areas occupied by the growths were perfectly circular, an assumption not in accord with the facts, but of sufficient validity so that the results might be tested against

the more accurate data obtained with the projectoscope and planimeter. The average radius of any given lot of transplants was calculated as follows:

$$r = \frac{\text{Sum of all axes}}{4 \times \text{number of transplants}}.$$

The approximate average areas could be calculated from the formula  $\pi r^2$ ; and from these areas the increments of growth could be determined. Comparative increments of growth, on the other hand, could be estimated directly from the squares of the radii as indicated in the formula  $\frac{r_z^2 - r_o^2}{r_o^2}$  where  $r_o$  is the average radius of the original transplant, and  $r_x$  is the average radius on any given day. The effect of any given experimental environment, as in the case of the measurements made with the projectoscope, was calculated by the formula

Increment under experimental environment
Increment under normal environment

### RESULTS

The comparative results with the two methods are shown in Table I, where a surprisingly close agreement is seen in most instances, particularly in the case of older growths in which the new cells occupied a more nearly circular area than in the first 2 or 3 days after transplanta-A comparative index of more than 1 indicates that the experimental environment was more favorable than the normal one, and conversely an index of less than 1 shows the experimental environment was less favorable. In no case was the sign of the index different by the two methods; and in only part of one experiment (T-68) did the indices vary widely. In this case the observer was making his first ocular micrometric measurements; and the growths during the first few days showed marked irregularity so that calculations made on the assumption of a circular growth would not obtain. When, in the later days of the experiment, the growth became more circular and the observer more experienced, the indices agreed more closely. By calculating the initial areas of the transplants according to their true shape—square, triangular or circular—doubtless the two sets of figures could be brought into closer agreement.

In recording the rates of migration and growth of cells from explants of spleen and buffy coats of blood we have found the ocular micrometer especially useful, because the areas occupied by different types of cells could be more accurately measured on account of the possibility of

TABLE I

Experi-	Dart	Medium	Mathod of antenning						Day				
No	1 11		זאפוחסת סו פצונשכווסם		1	2	3	4	25	٥	7	8	6
T-64	a	Normal plasma Plasma removed	Projectoscope	Increment "	0.6	3.1	8.1		12.0 32.0				
	,	Mormal along		Ratio $\frac{a}{b}$	99.0		0.53	1	0.37				
	o e	Plasma removed	Ocular micrometer	Increment "		1.2	4.0	7.2				`	
			-	Ratio $\frac{a}{b}$		0.58	0.53	0.52					
T-66	a b	Tb plasma Normal plasma	Projectoscope	Increment "		1.2	3.6 10.6	8.6 18.5		16.7 32.6		32.2 50.0	
				Ratio $\frac{a}{b}$		9.0	0.36	0.46		0.5	<del></del>	0.65	
	a b	Tb plasma Normal plasma	Ocular micrometer	Increment "		1.7	3.4	6.4		20.0 30.0			
				Ratio $\frac{a}{b}$		0.63	0.58	0.36	***************************************	99.0		<del></del>	
T-67	<i>a</i>	Tb plasma Normal plasma	Projectoscope	Increment		0.58		5.3	5.0		11.3 18.8		
	9	Tb plasma Normal plasma	Ocular micrometer	Ratio $\frac{a}{b}$ Increment		0.39	0.78	0.42	0.48		0.6		
				Ratio $\frac{a}{b}$		0.46	0.42		0.63		0.73		

49.5	11.0	2.9	16.0	10.4	2.8	0	15.6	0	?: 	27.0	29.9	0.0							
30.6	13.5	2.3	26.2	0.11	2.5	21.0	2.4.4	5	0.89	16.2	18.8	0.86		11.3	-10°0;	0.28	10.1	39.8	0.25
														2.3 5.0	16.5	0.27 0.32	4.1	16.4	0.25
_							0 % 0 10	1	0.57 0.76	7.	7.4	1.0		2.3	8.3	0.5			
2	2.8	2.2	7.25	5.25	1.38		7 +	:	0.57								0.78		0.28 0.27
\ -	0.8	1.86	1.83	0.63 5.25	2.8	,	2.1	- -	1.0	2.6	2.5	1.15 0.96		0.58	1.5	0.39	0.31	1,1	0.28
							t 0.45 2.		1.18	9.0	0.52	1.15		-					
	norement,	Ratio a	Increment	×	Ratio a		Increment		Ratio =			Ratio 2	<b>S</b>	Increment	×	Ratio -	Increment	,,	Ratio a
	Projectoscope		Total a solution	מ מוניוסטוריבי			Projectoscope	<b>:</b>		Ocular micromotor	10 maronical 11 ma			Projectoscope	"		Oculas micromotor		
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	T:63						T-31							77.1.					

obtaining a microscopic picture of each cell simultaneously with the measurements. The fact that these migrating cells quickly occupy a circular area also tends to make the two methods agree more closely than in the case of fibroblastic growths.

Another advantage of the ocular micrometric method is the possibility of placing the microscope in a warm box during the period of measurement, and thus one may avoid chilling the cultures and retarding their growth.

#### DISCUSSION

In presenting the ocular micrometer measurements as a basis for the calculation of growths of tissue cultures we do not mean to imply that it is as accurate as those given with the projectoscope and planimeter, nor do we think that the ocular micrometer has not been previously employed in examining tissue cultures, since remarks are encountered in the literature indicating that it has been used. In neither Fischer's book (2), in Levi's article on technique (3), nor elsewhere, have we found that approximate areas calculated from measurements of axes have been used for estimation of increments of growth or for comparative indices. While the simple inspection of growths will often give a fairly correct idea of the influence of a particular environment on the growth of cells in vitro, in many instances, where the culture media is composed of complex substances, it becomes necessary to keep quantitative records of the increment of growth if one is to estimate the effect of the components in the medium. The demands for quantitative data are even more pressing as one multiplies the number of pieces examined and the number of factors in the environment. Because the results of estimating the areas, and especially the comparative growth indices, were in such close agreement we feel that where the more complicated apparatus is not available one is justified in employing a method requiring simply an ocular micrometer and slide rule in addition to the usual material demanded for tissue cultures.

#### SUMMARY

In estimating the increments of growth of tissue cultures and of comparative indices it is possible to use areas calculated from measurement of two axes made with an ocular micrometer in an ordinary microscope. On the assumption that the growths are approximately circular in outline the squares of their radii are used as the factors for comparison. Results obtained with this method agree fairly closely with those derived from projectoscopic drawings and planimetric measurements.

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#### RABBIT POX

# I. CLINICAL MANIFESTATIONS AND COURSE OF DISEASE By HARRY S. N. GREENE, M.D.

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PLATES 19 TO 21

(Received for publication, June 26, 1934)

In December, 1932, a disease presenting many of the characteristic features of small pox in man appeared in a colony of rabbits maintained for the study of problems of constitution and quickly assumed epidemic proportions with a high mortality in certain classes of animals. The nature of the disease and the origin of the outbreak were obscure, but the picture presented indicated that the disease was due to a virus and not to a bacterial infection. This impression was supported by observations made in the spring of 1930 when there was a small epidemic of a disease presenting the same clinical and pathological picture.

While it is known that infection with vaccine virus may be transmitted from one rabbit to another by contact or to other animals in the same room, the prevailing impression is that these infections usually are mild or asymptomatic. However, a number of severe epidemic infections have been attributed to neurovaccine virus. In the present instance, work with a number of viruses was being carried on in several laboratories, but at the time very little information could be obtained concerning the use of vaccine virus. It was subsequently learned, however, that prior to the outbreak of the epidemic in question, at least three laboratories had inoculated rabbits with vaccine virus and that in two of these neurovaccine had been used. Some of the inoculated animals were on a lower floor of a building in which a small section of the breeding colony was kept, while others were on the same floor of the building in which the main colony was housed and there were connecting links by means of which a highly contagious infection might be transported from one group of animals to another, either in the same or in different buildings. If dissemination of vaccine virus occurred among the experimental animals in these laboratories, it apparently was not detected before the outbreak of this highly contagious disease in the breeding colony.

It seemed desirable to investigate this disease as fully as circumstances would permit. The colony in which the epidemic occurred is composed of pure bred and hybrid stocks of many breeds, and at the time of the outbreak contained more than 1,400 rabbits. Some were normal vigorous animals bred to standard specifications, while others showed or were known to transmit various physical and functional abnormalities. The colony had been under systematic observation for several years. The pedigrees and life histories of all animals were known, and the racial, familial and individual peculiarities of the stock had been made the subject of extensive investigation so that an unusual opportunity was presented for the study of the epidemic from several points of view.

Clinical, pathological and epidemiological studies of the spontaneous disease were carried out by the author; etiological studies were made by Dr. Pearce, Dr. Rosahn and Dr. Hu. All of this work was restricted by the necessity for the preservation of as much of the colony as possible, the use of measures to prevent the spread of the disease within the colony as well as to other stocks of rabbits, and by the lack of adequate facilities for the systematic management and study of an epidemic of such extensive proportions. Despite these handicaps, a fairly complete series of observations and experiments was carried out.

Investigations showed that the disease had been present in other stocks of rabbits for some time before it appeared in our breeding colony; later it was found in still other stocks, including animals under experimental observation, normal animals in the general stock room from which animals are obtained for experimental purposes, and in the Institute breeding colony. It is evident, therefore, that information concerning this disease is not only of medical interest, but is also of interest to those who breed rabbits or use them for experimental purposes. The reports to be submitted are intended to cover these several fields of interest, but the spontaneous disease and the epidemic have been studied with especial reference to the rôle of predisposing and constitutional factors for which the material is peculiarly appropriate.

A brief summary of the outstanding features of the disease has been published. The purpose of this paper is to present the clinical studies in more complete form. This will be followed by papers dealing with the pathology of the disease and an analysis of the results obtained from a study of the epidemic. Experiments dealing with the transmission of the infection, the disease produced by inoculation and the identification of the infectious agent will be reported by Pearce, Rosahn and Hu.

#### Materials and Methods

Study of the spontaneous disease in its several relations was based on a stock of between 1,400 and 1,500 rabbits. A detailed classification of these animals will be given in connection with the epidemiological studies. For present purposes, it is sufficient to say that the colony contained pure bred and hybrid stock from sixteen breeds including males and females ranging in age from several years to new-born young. There were males in active service and others in reserve, while the females included resting, pregnant and nursing does. There were young of all ages and many litters were born during the epidemic.

In the routine conduct of the colony, every litter is examined at birth and a record made. This is followed by a daily inspection of all young and of all nursing does for the purpose of noting any unusual development or any condition which requires attention, and the entire colony is examined once a week. These observations are made by members of the staff, but in addition the caretakers have been trained to note and report anything unusual, thus affording a double check on the condition of the colony from day to day.

Early in December the colony showed evidence of deterioration. Breeding operations were suspended, and a careful search was made for cases of illness including this particular disease. It was 2 weeks, however, before the first case was found. From this time on, cages containing infected animals were noted and all possible precautions were taken to prevent spread of infection until the disease became so widespread that further precautions appeared to be useless. However, strict isolation of the colony was maintained for about a month after the last case of the disease developed and all external lesions had healed.

During the epidemic, dead animals were collected daily for record and autopsy, cages containing young animals were inspected and obvious new cases of illness were recorded; sections of the colony were examined carefully day by day, especial attention being devoted to those animals which hitherto had shown no signs of illness. In this way, the entire colony was covered about twice a week and we were able to determine with reasonable certainty which animals, if any, had escaped infection. In the handling of supposedly uninfected animals, hand disinfection was carried out.

<sup>1</sup> Greene, H. S. N., Pecc. Soc. Exp. Biol, and Med., 1933, 20, 892.

As a part of the routine management of the colony, all dead animals are autopsied. During the epidemic a special effort was made to distinguish between deaths due to rabbit pox and deaths due to other causes, to note conditions peculiar to rabbit pox and complications associated with the disease. The pathological material obtained in this way was supplemented by material from animals killed during all stages of the infection, including the period of recovery, and by material from animals presenting complications and sequelae of various kinds, especially those which persisted or developed after apparent recovery from the pox infection.

Experiments were carried out to determine the length of the incubation period, the probability of acquiring the infection by the contact of normal with recovered animals, by the mating of normal with recovered animals and by offspring from recovered parents. Experimental tests were also made to determine the extent of the effect produced on the reproduction capacities of both males and females.

## Signs and Symptoms of Disease

The disease designated as rabbit pox occurred in two distinct forms, the one an asymptomatic affection which was recognizable only by the presence of characteristic lesions, while the other presented definite symptoms of an acute infection. It was found, however, that in either case the diagnosis could not be made with certainty except by careful examination and the detection of certain distinctive lesions. The lesions found on examination of living animals are divisible into four groups: (1) lymphadenitis, (2) papular lesions of the skin and mucous membranes, (3) keratitis or ophthalmia and (4) orchitis. These will be described in the order given.

Lymphadenitis.—The lymphoid system was invariably affected and usually the first definite sign of infection was enlargement and induration of the popliteal lymph nodes. The nodes of the inguinal region and anterior triangle of the neck were also consistently enlarged, and the lymphoid masses of the pharynx were usually swollen and in many cases filled the greater part of its posterior portion. In other regions, enlarged nodes were occasionally found but, as a rule, no appreciable change could be detected by palpation. Lymphadenitis persisted throughout the disease and in some animals was the only sign of infection.

Lesions of Skin and Mucous Membranes.—A macular rash preceded the appearance of papules and occurred either as the first sign of infection or a day or so after the discovery of enlarged popliteal lymph nodes. The rash was visible on those parts of the body not covered by hair and could be demonstrated elsewhere by clipping or shaving.

After a variable time papules erupted in the macular areas (Figs. 3 and 4). They first appeared as extremely small elevated swellings and in some cases re-

mained hardly visible or palpable throughout the infection. In other cases, growth occurred in an irregular manner and all variations in size up to nodules a centimeter in diameter were present in the same animal. They eventually became umbilicated, dry and covered with crusts. The vesiculation and pustule formation characteristic of small pox did not occur. Crops of macules and papules appeared in succession, and all stages of papular development could be found in a single area as in chicken pox. In severe infections, there were hemorrhages in the skin and papules tended to be hemorrhagic.

Papules were usually scattered irregularly through the skin of the entire body and were detectable with the greatest ease in the ears (Figs. 5 to 7), the lips, the eyelids and brows, the nape of the neck, the skin of the trunk and the scrotum (Fig. 17). Occasionally they occurred as confluent masses, but more commonly, although arranged in concentrated groups, individual lesions remained discrete. Such distributions were usually associated with an abnormal looseness of the coat and affected areas could be plucked bare with little effort.

Papules were also common on mucous membranes and often occurred in confluent masses associated with extensive edema. They were found on the lips and inner surface of the cheek in most cases of severe infection (Fig. 9), and when confluent presented a striking picture. The edema usually involved neighboring tissues and caused an intense swelling (Fig. 10) which, in some cases, became hard and indurated and persisted long after other signs of infection had disappeared. Such areas often became necrotic and sloughed with the production of large defects which healed with scar formation and resulted in permanent disfigurement (Figs. 19 and 20). The tongue and palate were frequently the seat of hard nodular infiltrations (Fig. 15), the centers of which often became necrotic and sloughed with the formation of deep ulcers. Occasionally the gums showed a diffuse grevish swelling with localized areas of necrosis extending to the roots of the teeth, which caused permanent damage or loss of teeth in recovered animals. These lesions were usually accompanied by a clear blood-stained secretion or a mucopurulent discharge from the nose, suggestive of snuffles (Fig. 8), which dried with the formation of thick, brownish, adherent crusts.

Papules were also found about the anus, on the vulva of the female and on the sheath in the male. In these situations they usually occurred as discrete lesions. Occasionally they were confinent and accompanied by edematous swelling which caused obstruction of the urethra and retention of urine, and sometimes resulted in necrosis of the parts.

An extensive edema of ears, legs, genitalia and tissues about the eye was also of common occurrence when such parts were severely affected.

Eye Lesions.—Eye involvement was of almost constant occurrence and resulted in a variety of lesions (Figs. 11 to 14). Many cases of infection showed only a slight marginal blepharitis with excessive lacrimation, which usually accompanied the macular rash but sometimes preceded it. In some cases, infection spread to the lacrymal duct and a chronic dacryocystitis resulted. Diffuse keratitis with

corneal ulceration was common. Iritis and iridocyclitis occurred later in the course of infection and were often followed by secondary glaucoma. In many animals, the occurrence of those individual lesions was masked by an outspoken purulent ophthalmia. Typical eye lesions were occasionally the only visible sign of infection.

Orchitis.—In males, the testicles usually showed a nodular or diffuse orchitis with edema of the scrotum (Figs. 16, 17). The involvement in many cases was so extensive that sterility of animals seemed inevitable. Lesions resolved, however, with restoration of fertility in most instances.

## Clinical Course of Disease

Incubation.—During the epidemic, it was impossible to fix the time of exposure of a given animal or of animals in a given cage, so that the incubation period could not be determined with accuracy. However, observations made on cage groups showed that there was considerable variation in the interval between the development of the first and subsequent cases of infection in the same cage. During the early stages and at the height of the epidemic, there was rarely more than 2 to 3 days between the development of the first and last cases, and not infrequently all of the animals came down at the same time. As the epidemic progressed, the irregularity increased until, in the terminal stages, the interval between known exposure and the appearance of definite lesions was frequently as much as a week and occasionally as long as 2 weeks.

In order to determine the incubation period with greater accuracy, two experiments were carried out during the terminal stages of the epidemic. In each case, six rabbits 2 months old and of the same litter were exposed to infection in the following manner. In one experiment the six rabbits were placed in a clean cage with a rabbit of about the same age which showed an active infection, and left for 24 hours after which the infected animal was removed. In the second experiment the animals were placed in a cage of the same size in which all except one of a group had died. The survivor was in the terminal stages of the disease and died in less than 12 hours.

There was no decided difference between the results of the two experiments. Most of the animals came down between the 5th and 7th days after exposure. The shortest incubation period was about  $4\frac{1}{2}$  days and the longest 9 days. It seems probable that during the

height of the epidemic the average incubation period may have been shorter. On the other hand, it is known that, during the terminal stages, the incubation was as long as 2 weeks in some instances, and may have been longer.

Symptomatology.—Constitutional symptoms of infection varied both in occurrence and severity, dependent upon breed, age and physiological status of affected animals, and, as has been pointed out, were frequently absent. A full account of this variation will be found in the report dealing with epidemiological studies.

In typical cases the onset was marked by an elevation of temperature which in some instances was as high as 106°F., and animals were obviously ill. Apathy and loss of appetite were early symptoms and rapid prostration was not uncommon. Diarrhea was common in the early part of the epidemic, both in young animals and in adults, but was infrequent later and its occurrence in the early stages of the epidemic was probably influenced by a coexisting dietary disturbance.

Symptoms resulting from involvement of individual organs were more constant. Infection of the upper respiratory tract was accompanied by labored respiration, mouth breathing and extreme weakness. Photophobia was commonly associated with lesions of the eye and could be recognized by partial closure of the lids. Animals with extensive lesions of the mouth and tongue generally refused food, probably more because of the pain caused by eating than from actual loss of appetite, and rapidly became emaciated.

Nervous system involvement was manifest in some animals by incoordinate muscular movement, fibrillary twitching and nystagmus. Paralysis occurred in other instances and was usually limited to special muscle groups. The sphincter muscles were most commonly affected, and such cases showed dribbling of urine and frequent passage of loose fecal material which adhered to the perineum, hardened and, unless removed, blocked the anus and urethra, causing retention of urine and feces. Generalized paralysis was not common and usually occurred in the terminal stages of a severe infection.

Types of Disease.—The majority of affected animals showed generalized lesions and symptoms referable both to systemic infection and involvement of individual organs. In many instances, however, lesions were more prevalent or conspicuous in a particular organ or part and although strict localization seldom occurred, this inequality allowed a basis for recognition of other disease types (Figs. 1, 2). Thus, in some cases the eruption in skin and mucous membranes constituted the clinical picture, while in others glandular enlargement, orchitis or ophthalmia was the only external manifestation of infection.

The limitation in distribution did not affect the severity of lesions, for they were no more pronounced than when associated with lesions of other parts. A purulent ophthalmia often persisted in an animal as the only sign of infection. On the other hand, a slight inflammation of the lids without lesions elsewhere was of equally frequent occurrence.

Symptoms of constitutional disturbance bore little relationship either to distribution or severity of external lesions. The mildest cases of ophthalmic, genital and cutaneous infections were usually asymptomatic, but occasionally showed the severest signs of ill health. Moreover, animals with extensive lesions of these parts often appeared in perfect health. Asymptomatic cases were also common both in animals with widespread lesions and in others in which lesions were so poorly defined that their presence could only be detected by the most careful examination. In respect to mortality, however, such cases were as serious as others, and at autopsy animals showed typical changes in internal organs.

Mild and abortive types of infection were common during the terminal stages of the epidemic, and a large proportion of animals of the most susceptible breeds and age groups survived infection contracted at that time.

Effects in Pregnant and Lactating Does.—In pregnant and lactating does, infection produced the severest symptoms.

Such animals were extremely ill and presented the characteristic facies of the disease, hunched in a corner of the cage in a stupor, breathing stertorously through nostrils obstructed by thick brownish crusts, lids partially closed over dull, lusterless eyes or glued together by dried secretions and, when forced apart, revealing a diffuse keratitis or destructive ophthalmia. Pregnancy was commonly terminated by abortion and marked improvement usually followed. Abortion was also frequent in non-infected, apparently healthy does, both in the latter part of the pre-epidemic period and during the epidemic. Young born at term were usually either dead, abnormal in some respect and non-viable, or deserted by the doe. Clinical or pathological signs of infection were never found in feti or young born dead. Lactating does were even more seriously affected and unless relieved of the strain of nursing, rapidly deteriorated and died. The separation of young from nursing does often improved their condition and, in many instances, brought about rapid recovery.

Complications.—Some complications were of such frequent occurrence and so intimately associated with the disease that they have been described as signs of infection. Thus, corneal ulceration occurred in most animals showing eye involvement and was often followed by perforation, iridocyclitis or glaucoma. Chronic inflammation of the lacrymal duct with mucocele was occasionally observed. Laryngitis was a constant complication of upper respiratory infections and was frequently accompanied by edema of the glottis which caused the stertorous breathing characteristic of many cases. Infection of the submaxillary glands was common and occasionally resulted in visible enlargement.

In many cases death was apparently due to a terminal bronchopneumonia which was a frequent complication and in some instances may have resulted from the aspiration of foreign material due to diminished sensibility of the larynx. Pulmonary abscess was less common but was the direct cause of the majority of postepidemic deaths. Other secondary pyogenic infections were rare. Dental abscess occurred in a number of animals and in a few instances involved the bone of the jaw.

During the early part of the epidemic the disease was frequently complicated by gastrointestinal disorders. Diarrhea has been mentioned. In young animals, the discharges consisted of thick tenacious mucous but in adults were usually made up of loose watery fecal material. Intestinal obstruction caused by impactions in the cecal region was also common. These disorders were of frequent occurrence previous to the epidemic and are not attributable to the disease. However, they were serious complications and when superimposed upon the infection, usually resulted in death.

Mortality.—Death might occur within a few hours after infection was first noted or be delayed for days or weeks. The total mortality was 46.4 per cent but showed wide variations in different age groups and breeds. Among animals less than 14 weeks of age, 71.8 per cent of cases of infection died; the mortality rate was highest among those 4 to 8 weeks old. In adults, although the mean mortality was about 15 per cent, the rate in different breeds varied widely, certain lines being almost completely wiped out while in others no deaths occurred.

A full discussion of the mortality will be reserved for the report concerned with the epidemiological features of the disease.

Receivery.—Many animals with severe infections recovered (Fig. 18).

Prognosis based on the nature of symptoms and type of disease sometimes proved correct, but as a rule no indication of the final outcome of infection could be obtained from clinical examination.

In cases of recovery, lesions underwent slow resolution extending over a period of weeks, and in some instances healed with the formation of scars. Reactivation and reinduration of skin papules with the development of new lesions was occasionally noted after healing was well advanced, but it is not certain that relapse occurred.

Sequelae.—All recovered animals bear stigmata of the disease. Corneal scars and scarred hairless areas in the skin of the ears (Fig. 21) have persisted to the present time. Many animals were permanently disfigured by the healing of large defects by secondary intention and subsequent contracture (Figs. 19, 20). In other instances, the sloughing of portions of the genitalia rendered the animals unfit for breeding service.

A variety of abnormalities of the incisor teeth resulted from gum lesions and dental necrosis. In many animals both primary and secondary incisors were completely destroyed, and in others regeneration resulted in malalignment which led to direct antagonism with loss of cutting edge or to an outspoken deformity in which antagonism was entirely lost and growth, therefore, unrestrained.

Although to outward appearances animals were sufficiently recovered and fit for the resumption of breeding 2 months after the beginning of the epidemic, the occurrence of two widespread disorders later in the spring indicated that not even at that late date had they reached a normal level of general health and vigor. Although a thorough investigation of these conditions could not be made, they appeared to be attributable in part at least to the reaction of convalescent and constitutionally weak animals to adverse nutritional or other environmental factors and, in this sense, were sequelae of the pox epidemic.

The first disorder to appear was characterized by jaundice, progressive weakness and emaciation. After prolonged illness most adults improved or recovered, but the disease was usually fatal in young rabbits. Those that died were autopsied and showed marked cirrhosis of the liver of mixed type, entirely different from the cirrhosis due to coccidiosis. The second condition was characterized by paralysis of the hind quarters, atrophy and degeneration of the fibers of the mas-



and parturient females, and at autopsy all showed similar lesions consisting chiefly of changes in the liver comparable with those found in the toxemias of pregnancy. It is noteworthy that these were all recovered animals and showed large, persistent scars in the liver, but the exact relationship of the disorder to previous pox infection is uncertain.

#### Carrier Transmission

Following the epidemic many seriously affected animals were discarded. However, a selected group was retained for tests of carrier transmission which were performed simultaneously with tests of reproductive functions. Normal animals were mated with recovered animals, recovered males with normal females and recovered females with normal males, but in no instance was a mating of this kind followed by the development of disease. As a further test, normal animals of the most susceptible age groups were imported into the colony and were kept in continuous contact with recovered animals but showed no sign of infection. Moreover, approximately 500 litters containing over 2,500 animals have been born from recovered animals since the end of the epidemic. All of these young have been examined clinically and many have been subjected to postmortem examination, but in no case has any indication of the transmission of the infection been found.

## Contagiousness of Infection

A comparison of the present epidemic with that of 1930 indicates that the contagiousness of the infection may vary greatly in different epidemics. In 1930 only a small part of the colony contracted the disease. Spread of the infection was effectually checked by prompt isolation or killing of animals which developed the disease, as was indicated by the fact that there were no immunes among the survivors of the first epidemic. In the present instance, however, isolation of rooms with systematic disinfection and removal of infected or apparently healthy animals to other quarters had no appreciable effect on the spread of disease.

At the beginning of the outbreak, infection invariably followed exposure and spread was so rapid that within 2 weeks the distribution

age of the lesion, for marked variation in size is also found among papules of equal maturity. Many of these lesions are hemorrhagic.

Fig. 4. Skin of a pregnant Himalayan doe self-plucked for nest fur, showing both dry, crusted papules and others in earlier stages of development. Minute lesions of this type occurred in all adult Himalayans.

#### PLATE 20

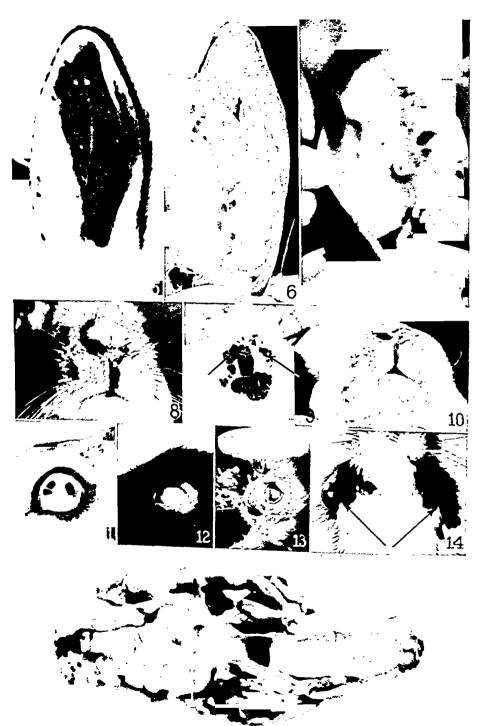
- Fig. 5. Transilluminated ear showing early macular lesions distributed in the more vascular areas.
  - Fig. 6. Ear at the height of infection showing typical papules.
  - Fig. 7. Ear showing unusually large papules with umbilication.
- Fig. 8. Sanguinous discharge from the nares in an acutely fatal infection. This was a frequent early sign of disease, and in the young Belgian rabbit shown in this photograph it was the only external sign of infection.
  - Fig. 9. Typical papular lesions of the lips.
- Fig. 10. Diffuse edematous infiltration of the muzzle in a young Rex rabbit. Compare with Figs. 8 and 9.
- Fig. 11. Eye showing diffuse clouding of the cornea. This was the only visible lesion found in this animal—a monosymptomatic infection with no constitutional symptoms.
  - Fig. 12. Panophthalmia with total destruction of the lens.
  - Fig. 13. Purulent ophthalmia with ectropion of lids.
- Fig. 14. Unilateral secondary glaucoma. A common sequel of ophthalmic infections.
- Fig. 15. Nodular lesions of the tongue and palate with infiltration of gum margins.

#### PLATE 21

- Fig. 16. Diffuse orchitis with edema of scrotum in an adult Belgian buck.
- Fig. 17. Nodular orchitis with papular lesions of scrotum in an American blue buck.
  - Fig. 18. Recovery with permanent impairment of vitality after severe illness.
- Fig. 19. Healing of a diffuse lesion with contracture resulting from necrosis and sloughing of a portion of the right upper lip.
- Fig. 20. Gangrene followed by sloughing of upper lips and nose in a young Rex. The incisor teeth show malocclusion resulting from the infection in which the upper and lower cutting edges are in apposition. Root infections with permanent injury to the teeth were common.
  - Fig. 21. Scarred hairless areas in the skin of the ears 1 year after recovery.



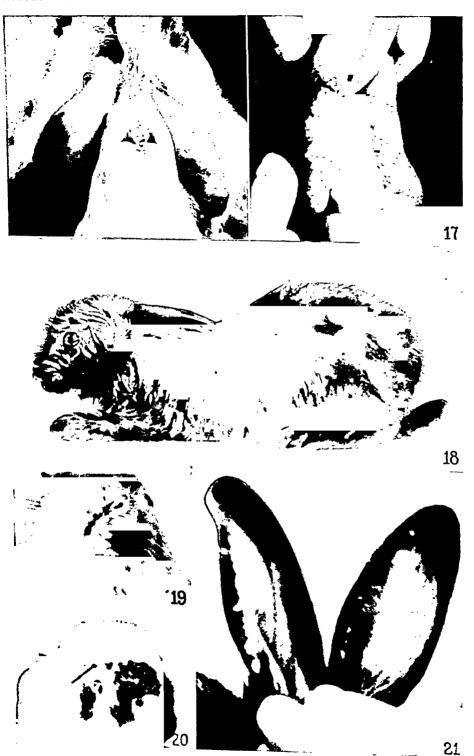








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#### RABBIT POX

## II. PATHOLOGY OF THE EPIDEMIC DISEASE

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PLATES 22 TO 24

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The clinical manifestations of epidemic rabbit pox have been described.<sup>1</sup> The purpose of this paper is to record the results of pathological examinations.

#### Material and Methods

The epidemic of rabbit pox was preceded by an increase in gastrointestinal and nutritive disorders and infections of the upper respiratory tract with a consequent increase in the number of deaths due to those causes. These conditions were prevalent during the epidemic so that animals coming to autopsy represented not only deaths due to rabbit pox, but also deaths due to other causes and to rabbit pox complicated by other conditions.

During the epidemic, postmortem examinations were made on over 900 rabbits. These included deaths from various causes and animals killed for the purpose of obtaining material representing all stages of the disease uncomplicated by other conditions. The methods employed in the collection of material were outlined in the previous paper. Tissues were fixed in 10 per cent formalin, Zenker's and Helly's fluids and sectioned in parafin. Sections were stained with hematoxylin and cosin; Giemsa's stain was also used in examinations made for inclusion bodies.

### Gross Postmortem Findings

The postmortem picture was not always characteristic and in some cases diagnosis was questionable, owing to the absence of distinctive macroscopic lesions or to the confusion created by other conditions prevalent at the time. As a rule, however, marked alteration in the gross appearance of tissues and organs and the occurrence of wide-spread focal lesions in the skin or elsewhere made identification of the disease possible.

<sup>1</sup> Greene, H. S. N., J. Exp. Med., 1934, 60, 427.

Areas of focal infiltration and necrosis were nearly always present and formed the most definite evidence of infection. Apart from the skin (Figs. 1, 2), mouth (Figs. 8, 9) and upper respiratory tract, they occurred with the greatest frequency in the liver, spleen and lungs, but were found in every organ of the body with the exception of the brain and kidneys.

The general appearance of animals dying from fulminating infections necessarily differed from that of animals in which the course of disease was more protracted. As a general rule, however, animals were well nourished with an abundance of fat in normal depots, and only in those cases associated with obstruction of the pharynx or with extensive ulceration of the tongue and mouth was extreme emaciation found.

Subcutaneous tissues were often markedly edematous and pea-sized nodules of necrosis were occasionally found in fat and connective tissue. Muscle and serous membranes rarely showed clearly defined focal lesions but small miliary hemorrhages were common (Fig. 3).

## Lesions within the Abdominal Cavity

The peritoneal cavity usually contained an excess of free fluid which in rare cases was blood-stained. Small areas of fat necrosis were common in the omental, mesenteric and retroperitoneal fat, and occasionally characteristic pearly white nodules of pin-head size were scattered over the surface of the peritoneum.

Stomach and Intestine.—The stomach was usually filled with food, and in many cases, although autopsy quickly followed death, digestion and perforation of the walls had occurred. Small petechial hemorrhages were occasionally found on the surface of the cecum and small bowel, but otherwise focal lesions were extremely rare.

In some cases, the intestines were distended with fluid and gaseous material and the anal region stained with diarrheal discharges. In others the cecum and upper portion of the colon were filled with masses of fecal material which were hard and dry or of a stiff, putty-like consistency. These masses were of variable extent and usually they were separated by accumulations of a thick tenacious mucoid material. Occasionally, this condition was found in the lower portion of the small as well as in the large intestine. It was also present in animals dying of other causes before and during the epidemic so that it cannot be regarded as peculiar to rabbit pox. It is rather evidence of an abnormality connected with the epidemic and accentuated by pox infection.

Liver.—The liver was always involved and usually had a characteristic appearance. In typical cases it was moderately enlarged, firm in consistency and pale yellow or yellowish brown in color with a profusion of translucent pearly white or opaque grey nodules a millimeter or more in diameter scattered over its surface and distributed throughout the organ (Fig. 5); nodules were occasionally found in the wall of the gall bladder. In some instances, these small lesions were accompanied by larger irregular areas of necrosis measuring as much as 0.5 cm. in

diameter. In other cases the liver was markedly enlarged and showed an extreme degree of yellowish red mottling suggestive of passive congestion. In fulminating cases, characteristic nodules were usually absent.

Spleen.—The spleen was invariably affected, and as a rule was moderately enlarged, dark red in color, firm and showed a few small areas of necrosis comparable with those found in the liver (Fig. 6).<sup>2</sup> The greatest enlargement occurred in fulminating cases and in those associated with secondary infection. Under these conditions the spleen was soft and considerable blood and pulp could be scraped from its cut surface. Exceptionally, the organ was normal in size, a dirty greyish brown in color and showed numerous focal lesions.

Kidneys and Adrenals.—The kidneys were enlarged and pale but characteristic macroscopic lesions were never demonstrated. The adrenals were usually large and greyish yellow in color; they were soft and showed a loss of architectural definition suggestive of autolysis, but occasionally distinct areas of focal necrosis

Genitalia.—In the female the ovaries and uterus were often riddled with focal lesions. Infection of the uterus was frequently accompanied by a purulent exudate and abscesses were sometimes found in the wall. This was particularly true of were found. and abscesses were sometimes found in the main. This was particularly due of animals which had recently aborted or given birth to young. The testicles of the male were always enlarged, and in most instances contained numerous focal and diffuse areas of necrosis varying from minute nodules to lesions a centimeter or more in diameter. Edema and hemorrhage were present in some cases.

## Lesions within the Thorax

The pleural and pericardial cavities usually contained an excess of clear fluid and occasionally were markedly distended. Purulent exudates were not seen. Fecal lesions were common on pleural surfaces but rare on the pericardium.

Lungs.—The lungs showed a variety of conditions. Visible or palpable lesions were nearly always present but occasionally the lungs were normal in appearance. The most constant lesion was a small subpleural nodule, and in some instances these were peppered thickly over all surfaces (Fig. 4). In early and uncomplicated infections, these translucent or pearly white nodules were frequently the only lesions present. More advanced cases showed typically necrotic, miliary nodules and occasionally larger pea-sized areas of necrosis with irregular patches of consolidation (Fig. 7). These areas of consolidation frequently measured a centimeter or more in diameter on the pleural surface and extended into the underlying tissue of the lung as a conical or wedge-shaped mass. They were greyish yellow or a dirty greenish grey color and sometimes were surrounded by a narrow zone of

The spleens shown in Figs. 5 and 6 are from Belgian hares. The enlargement is more marked than was generally observed at autopsy and is due in part to a coincident abnormality of the spicen found among animals of this breed in our colony.

hemorrhage or congestion as in the case of an infarct. Occasionally these were the only macroscopic lesions found. Diffuse consolidation of one or more lobes was seen in some animals but was less frequent than other lesions.

The trachea and bronchi appeared normal in early cases. In more advanced cases, they usually contained an abundance of white frothy material which rarely was blood-stained.

Heart.—The heart was usually pale in color, soft and relaxed. In other instances it was normal in appearance. In a few cases marked distension of all chambers with fluid blood was noted. Rarely focal lesions were found in the myocardium.

Thymus.—The thymus was generally enlarged and occasionally markedly edematous. Petechial hemorrhages were of almost constant occurrence and focal areas of necrosis were common.

## Lesions of Miscellaneous Organs

Lymph Nodes.—Lymph nodes were not universally affected. The most pronounced abnormalities were found in the pharyngeal and deep cervical nodes and in the superficial nodes in general, especially the popliteals. As a rule, these nodes were swollen and edematous and frequently showed characteristic focal lesions; occasionally they were dark red in color or contained petechial hemorrhages. In contrast with this, characteristic changes were rarely seen in the thoracic and abdominal nodes. Occasionally they were slightly swollen and edematous but, as a rule, no gross alterations could be detected.

Bone Marrow and Periosteum.—The bone marrow was always abundant but showed many variations in color and consistency. Frequently it was reddish grey in color with small scattered hemorrhages and larger greyish areas of necrosis. Under such conditions it was generally of normal consistency. In many instances it was uniformly grey in color and cheese-like in consistency. Rarely it was dark red and fluid.

The periosteum often showed greyish red elevated thickenings interspersed with areas of hemorrhage. These lesions were most common in the periosteum of the flat bones of the skull.

Thyroid and Parathyroids.—The thyroid, as a rule, was small and pale and often showed focal lesions. Parathyroids were likewise generally small but focal lesions were much less common.

Brain and Spinal Cord.—The brain ordinarily showed no other abnormality than an engorgement of pial vessels. In a few cases there was a slight increase in the amount of cerebrospinal fluid, and in one animal the ependyma of the lateral ventricles was markedly granular.

The spinal cord was usually normal in appearance. In a few instances it was slightly swollen and edematous.

Submaxillary Gland.—The submaxillary gland was almost invariably enlarged and contained localized and diffused areas of necrosis.

## Microscopic Findings

Both in the gross and microscopically, the most distinctive lesion in all organs and tissues was the sharply circumscribed nodule or papule. More diffuse lesions arising either from the coalescing of adjoining nodules or the simultaneous affection of large areas were also common. Microscopically, the composition of these lesions varied somewhat with their location and age but, in general, affected areas showed comparable histological changes.

The fully developed nodule or papule was found to be made up of a central area of necrosis in which cell outlines and cell types were completely lost, surrounded by a zone of mononuclear cell infiltration. This zone and the neighboring tissues were usually edematous and occasionally hemorrhagic. In the more diffuse lesions the focal character was entirely lost, large areas of tissue were infiltrated, necrosis was widespread and edema was marked. Both types were found together in fatal infections, but among animals killed in early stages of the disease there was a notable tendency for the lesions of a given animal to be limited to either the discrete or the diffuse form.

Skin and Mucous Membrane.—Characteristic lesions of the various types were found in the corium and subcutaneous tissues. The epithelial changes characteristic of small pox in man were not found. In early lesions the epidermis remained intact and unaltered. Pathological changes were demonstrable only in those cases associated with large necrotic areas in the corium. These consisted in atrophy and degeneration and suggested secondary nutritional or pressure disturbances rather than the localization of the specific cause of the disease in the epidermis itself.<sup>3</sup>

Similar lesions were found in the mucous membranes of the mouth and pharynx but in this situation focal lesions were generally larger and diffuse edematous infiltrations were of more frequent occurrence.

A large amount of material from fatal cases of infection and from animals killed at earlier stages of the disease was examined with a view to determining the pathogenesis of focal and diffuse lesions. It was found that in both cases the lesions began as a vascular or perivascular process affecting blood vessels and lymphatics, but the devel-

In a recent epidemic of a disease presenting identical clinical signs and symptoms, the lesions of the skin were not limited to the corium, but were also found in the epidermis where they consisted of focal epithelial degeneration, vacuolization and vesicle formation. This disease was less contagious and far less fatal than that described in this report.

opment of the lesion was most clearly defined in the case of the smaller blood vessels. In the earliest lesions the endothelium of affected vessels was swollen and granular and occasionally detached. There was no apparent alteration in the walls of the vessel, but the perivascular tissues were slightly distended with a serous exudate and contained a few cells grouped in loose collar formation about the affected vessels. The cells were chiefly large mononuclears with some lymphocytes and occasional polymorphonuclear and red blood cells. There was also an appreciable tendency to the accumulation of leucocytes in the lumen of the vessel, and occasionally cells were seen penetrating the vessel wall.

As the lesion progressed, the process assumed either a focal or diffuse form. In the case of focal lesions (Fig. 14), the accumulation of cells increased both inside and outside of the vessel. The walls of the vessel showed hyaline changes and eventually the outlines of the vessel were lost. The cells at the center of the nodule thus formed became necrotic and disintegrated so that cell outlines and cell types were no longer recognizable. At this stage, the lesion was composed of a central area of necrosis surrounded by a zone of mononuclear cells of variable extent. This zone and the adjacent tissues were edematous as a rule, and occasionally hemorrhagic.

Lesions of this type varied from extremely minute granules to masses a centimeter or more in diameter. They were widely scattered or in close proximity to one another but, as a rule, the intervening tissues were little if at all affected.

Diffuse lesions differed from the focal in that, from the beginning, there was a more widespread involvement of blood vessels and lymphatics with less tendency to the concentration of cellular elements about affected vessels. In typical cases, cells and fluid were spread diffusely through the tissues (Fig. 13); mononuclear cells still predominated but there was considerable increase in the relative number of polymorphonuclear and red blood cells. Widespread edema and extensive necrosis of tissues were characteristic features of these lesions and hemorrhage was a frequent occurrence.

There was no truly pustular stage in the development of either focal or diffuse lesions. With the extension of the necrosis in the deeper layers of the skin, the blood supply to adjacent tissues was cut off or

impaired, giving rise to an anemic necrosis which frequently extended well beyond the limits of the specific lesion. In this way many focal lesions extended to the surface with the formation of ulcers covered by crusts, while confluent and diffuse lesions gave rise to mass necrosis or gangrene followed by sloughing of larger masses of tissue.

There was no evidence that a pyogenic or other secondary infection played any part in the production of these lesions. Focal and diffuse reactions had a common mode of origin, but from the beginning they pursued a different course. Evidently the difference was mainly one of degree and was referable to the extent of the vascular injury on the one hand, and the ability of the animal to limit the action of the causative agent on the other. As a rule, given animals showed a tendency to one type of reaction or the other, but with the progress of the disease focal lesions frequently became confluent and occasionally gave way to an outspoken diffuse reaction.

Lungs.—The lungs showed focal and diffuse lesions comparable in all respects with those of the skin. Circumscribed areas of mononuclear infiltration were found in the vicinity of small blood vessels (Figs. 12, 15) and occurred with the greatest frequency near the pleural surface of the lung, but were also situated deep in its substance and occasionally were scattered profusely throughout a section. They were rarely as sharply circumscribed as those found in the skin, and frequently alveolar walls extending from such foci were infiltrated and swollen with exudate. Occasional sections showed thickening and infiltration of alveolar walls with no clearly defined focal lesions. This infiltration was sometimes widespread but more often occurred in scattered patches without relation to the bronchi. More diffuse lesions in which large areas were infiltrated with an exudate rich in mononuclear cells and fluid and containing many polymorphonuclears were common in more advanced cases of infection, but were also found in association with early focal infiltrations. Older lesions showed varying degrees of degeneration, and in fatal cases total necrosis of an entire lobe was not uncommon. Lesions of this kind were comparable with the areas of mass necrosis seen in the skin. In rare instances, there were also extensive areas of consolidation composed mainly of polymorphonuclear cells which appeared to be due to the action of some secondary invader.

In early cases of infection the peribronchial lymphatic tissue was generally hyperplastic, but the bronchi themselves showed no pathological alteration. In many fatal cases, however, their walls were infiltrated and necrotic and their lumina plugged with exudate and desquamated epithelial cells. The absence of primary bronchial changes and the great frequency of focal interstitial lesions would suggest that the infection spread through blood and lymphatic channels rather than through bronchial passages.

Liver.—The liver was occasionally engorged with blood but, as a rule, showed in addition to the focal and diffuse areas of infiltration and necrosis only an extreme degree of cloudy swelling. Early foci were closely associated with blood vessels in the portal spaces (Fig. 10) but older and more diffuse lesions extended into the liver parenchyma and resulted in extensive destruction of lobules. Fatty degeneration was marked in some cases but was also a common finding in animals which showed no evidence of pox infection. In fulminating infections, typical focal necroses were not found and the microscopic picture was that of an acute diffuse degeneration and necrosis involving the whole organ. Individual cells were swollen, granular and indistinct with pyknotic nuclei, and in scattered areas were definitely necrotic. Mononuclear infiltrations were absent.

Splcen, Lymph Nodes and Bone Marrow.—The spleen showed a varying degree of congestion, which in some cases was extreme. The sinuses were distended and contained an excessive number of mononuclear cells; polymorphonuclear leucocytes were rare. Immature leucocytes and nucleated red cells were common. Malpighian corpuscles were generally enlarged, edematous and contained cells larger and more granular than normal; rarely the corpuscles were reduced in size. Focal and diffuse areas of necrosis were found in many instances. Focal lesions were most frequent on the surface but were also found in Malpighian corpuscles. Occasionally necrosis was widespread throughout the organ, the pulp consisting of a homogeneous mass of degenerating cells and pigment, while the Malpighian corpuscles were represented by nests of nuclear detritus.

Superficial lymph nodes were generally hyperplastic with large active germinal centers. Their increased size, however, was in large part due to edema. Sinuses were distended with fluid and individual cells widely separated. The deep cervical nodes were most markedly affected and frequently showed cystic dilatation of their medullary sinuses. These changes were often accompanied by discrete and diffuse areas of necrosis and, in many instances, entire nodes were necrotic.

The bone marrow in many fatal cases was involved in so widespread a necrosis that many sections failed to show a single normal cell (Fig. 11). In other instances small focal necroses were scattered throughout and hemorrhages were common. In less affected areas vessels were engorged with blood, large and small mononuclear cells were abundant, while polymorphonuclear cells were rare.

Miscellaneous Organs.—Cloudy swelling in the kidneys was so marked that many tubules were completely occluded. Other tubular or glomerular changes did not occur and necrotic foci were never found.

The adrenals often showed focal areas of necrosis. Widespread degeneration and disintegration were common in fatal cases but may have been of postmortem origin. In animals killed and immediately autopsied, however, similar but less marked changes were found. Diffuse areas of necrosis were of rare occurrence.

Discrete and diffuse areas of necrosis were common in the testicle. In early lesions there were scattered patches of mononuclear cells in the interstitial tissue and around the tubules. These increased and eventually became necrotic. In

some instances, the necrosis was limited to small areas, while in others there was

The ovaries were usually hyperemic and frequently showed focal lesions. Simple cysts were also common. The walls of the uterus often contained multiple foci of necrosis and abscesses were occasionally found.

Necrotic foci, areas of hemorrhage and edema were frequent findings in the submaxillary gland, thyroid and thymus.

The walls of the gastrointestinal tract were generally hyperemic with small, scattered areas of hemorrhage and enlarged lymphoid follicles, but typical necrotic foci were not observed.

The heart showed no singular microscopic changes. Vacuoles were occasionally found in muscle fibers but cellular infiltrations or necrotic lesions were absent in

In other regions of the body muscle fibers were frequently separated by edema, and diffuse cellular infiltrations, hemorrhage and necrosis were common.

Many sections were taken from different parts of the brain, but aside from engorgement of vessels, no definite pathological changes were found.

Vascular Lesions.—Vascular damage was intimately associated with all lesions and formed the focus for the cellular infiltrations characteristic of the disease in all organs and tissues. Early changes consisting of swelling and desquamation of endothelial cells were found in small vessels and precapillaries in the vicinity of fresh infiltrations. In focal lesions the immediate surrounding tissues were infiltrated with mononuclear cells which, in some instances, could be observed migrating through the vessel wall. In diffuse lesions endothelial damage was more intense and more widespread. Later stages were characterized by hyaline degeneration and necrosis of the vessel wall. But in the larger vessels no distinctive

Inclusion Bedies.—A thorough search was made for inclusion bodies in specially cut and stained sections. Many tissues from animals in different stages of infection were examined, but in none were cytoplasmic or nuclear changes found which

Epidemic rabbit pox is the most destructive contagious disease which we have encountered in the rabbit. Its extreme contagiousness and high mortality in certain classes of animals are comparable with the scourges of small pox in man and, in many other respects, the rabbit infection shows resemblances to this disease. In typical cases the characteristic features of small pox are duplicated and present the distinguishing signs of infection. The cutaneous eruption preceded by an crythematous rash and passing through the stages of papule. oustule and crust, the involvement of special organs, the complications and sequelae are similar in both diseases. The atypical forms of the two diseases also show similar modifications with lesions ranging from confluent eruptions to inconspicuous papules and systemic reactions varying from the complete absence of detectable symptoms to extreme prostration. The severity of the infection in pregnant and lactating females, the occurrence of abortions and the high mortality in young animals are especially significant.

Pathologically, the lesions in the skin of the rabbit were more deeply situated and resembled more closely those of the mucous membrane in man; they did not show the typical epithelial changes which have been described in the formation of vesicles and no typical inclusion bodies were found. In other respects the two diseases appear, both clinically and pathologically, to be essentially the same or identical. The absence of typical vesication and the failure to find inclusion bodies may be referable to the deep location and rapidly destructive nature of primary lesions. This assumption is borne out by subsequent experience with a less severe form of infection in which typical vesicles and inclusion bodies were present.

Characteristic lesions occurred with greatest frequency in the skin and mucous membranes of the mouth, nose and pharynx, in the lungs, liver, spleen, lymph nodes and bone marrow, and in the testicles and ovaries. In fatal cases of infection focal or diffuse lesions or both were nearly always present in these organs, but in some fulminating infections no lesions could be found in the skin.

Diffuse and focal lesions occurred together or as alternative processes, apparently depending upon the severity of the infection. In fulminating cases of disease, there was widespread degeneration and necrosis of cells comparable with those found in many infections and intoxications and in no way distinctive. The more common diffuse lesions, however, presented the characteristics of an acute inflammatory reaction in which extensive edema, a variable amount of hemorrhage, marked mononuclear but comparatively slight polymorphonuclear infiltration and widespread necrosis were distinctive features. Focal lesions consisted of clearly defined mononuclear infiltrations in which polymorphonuclear cells were almost entirely absent and edema and hemorrhage were less marked than in the diffuse form.

Focal lesions were intimately related to small blood vessels which usually showed varying degrees of endothelial damage. Diffuse lesions were also associated with vascular injury which, in cases not obscured by necrosis, was sometimes found widespread in small vessels and precapillaries. More frequently the damage was localized in single vessels, but was more intense than in focal lesions and accompanied by extensive edema and beginning necrosis in surrounding tissues, indicating that this type of diffuse lesion arose from extension of a focal process. In many fatal cases, however, necrosis was widespread and with loss of histological details, the original focal or diffuse nature of the lesion could not be determined. This was particularly true of pulmonary lesions which, in advanced cases, usually showed extensive consolidation with an exudate largely made up of mononuclear cells but also containing many polymorphonuclear cells and mass necrosis.

In view of the interest which has been manifested in the etiology of pneumonia in virus infections, it may be pointed out that lesions of this kind were not peculiar to the lungs. Similar processes were observed in the lesions of the skin which at times spread very rapidly with the development of a massive edema followed by necrosis and sloughing of the affected part. In these cases the exudate also contained a number of polymorphonuclear cells. Many of these animals survived, and it is worthy of note that the acute diffuse reaction was not followed by abscess formation as would be expected if the lesion were due to a secondary bacterial infection. Healing occurred as in the case of the typical focal lesion which supports the view that the diffuse reactions with edema, hemorrhage and infiltrations containing polymorphonuclear cells were produced by the action of the same agent which was concerned in the production of the focal lesions. From the information at hand, the action of a secondary invader cannot be excluded. At the same time, it must be remembered that the presence of a secondary invader does not prove that it played any essential part in the production of the lesion, and from the evidence available it appears that in this disease both the diffuse and the focal lesions were attributable to the same organism.

As a matter of fact, the diffuse reaction in the skin corresponds closely with the lesion usually obtained by a cutaneous or intracuta-

neous inoculation of vaccine virus, and any doubt concerning the etiology of pulmonary lesions is equally applicable to lesions of the skin, including those produced by inoculation with bacteria-free filtrates as well as the lesions seen in spontaneous infections.

Other lesions to which attention should be directed are those of the hematopoietic system, which in many fatal cases were so extensive that complete destruction of individual organs resulted. In such instances the spleen, bone marrow and many lymph nodes were so necrotic that microscopic identification depended rather on general outlines than on histological detail. The most constant finding, aside from the widespread necrosis in these organs, was the almost complete absence of polymorphonuclear cells. The sinuses of the spleen contained numerous mononuclear leucocytes but other forms were scanty, and in the bone marrow although their mononuclear antecedents were plentiful, polymorphonuclear cells themselves were extremely rare.

The remarkable healing of lesions of this disease in recovered animals is also worthy of mention. This was particularly noticeable in the organs of generation which, although most extensively affected, were repaired with restoration of spermatogenesis and ovulation. Cases were numerous in which clinical examination indicated universal and profound parenchymal damage. Yet such animals rapidly recovered normal functions and, in less than a month, were capable of active breeding service, and in general behavior could not be distinguished from normal animals.

Small pox was once regarded as among the most serious menaces to man, and in recognition of this fact protective measures were instituted which are unsurpassed by those employed against any other disease. Rabbit pox is clearly a serious menace to the rabbit and unless adequate precautions are taken, may seriously reduce the usefulness of the rabbit as an experimental animal. Typical cases of the disease are as easily detected as typical cases of small pox, but atypical and asymptomatic infections might escape detection or be mistaken for snuffles or for some innocuous disease. But because of their insidious and highly contagious nature they are a highly dangerous form of infection. Careful examination will, however, reveal sufficiently definite signs of infection in all forms of the disease to arouse suspicion or to make a positive diagnosis. From previous experience, it also

appears that if sporadic cases of infection are detected before the disease has become epidemic, the infection can be suppressed by the destruction or isolation of infected and exposed animals. Such measures are apparently of no avail during the height of an epidemic.

The probable origin of the epidemic will be discussed in a subsequent paper. It may be said, however, that available evidence indicates that the infection which was responsible for the present epidemic was not introduced from the outside but originated in the Institute and was either masked by some other disease or spread in atypical form and thus escaped attention until it reached the breeding colony as an outspoken epidemic disease.

Finally, clinical and pathological evidence lead to the conclusion that rabbit pox is comparable with small pox in man and that it is produced by an agent closely related to the virus of small pox.

### SUMMARY AND CONCLUSIONS

The lesions found in animals with epidemic rabbit pox have been described in this paper. The most distinctive gross lesion in all organs and tissues was the small nodule or papule which was found to consist of mononuclear infiltration and necrosis. Diffuse lesions were also found in which the infiltration was widespread and accompanied by edema, hemorrhage and extensive necrosis of affected tissues and organs. The possibility of the diffuse lesions being due to the action of secondary invaders was considered, but available evidence indicated that the different types, including pneumonia, represented reactions to a single causative agent. Moreover, an intimate relationship was observed to exist between lesions and small blood vessels in which primary endothelial damage was usually apparent. The degree of vascular damage generally corresponded to the extent of the lesion and it is probable that this in turn corresponded to the dose of the causative agent.

The close analogy between the clinical manifestations and pathological processes of this disease in the rabbit and small pox in man led to the conclusion that the disease in the rabbit is essentially the same as small pox, and that it is probably produced by a virus closely related to the virus of small pox. Available evidence indicated that the infection originated in the Institute and that it spread in atypical form or

masked by some other disease until it reached the breeding colony as a clearly defined epidemic infection.

### EXPLANATION OF PLATES

### PLATE 22

- Fig. 1. Undersurface of the skin from a fatal case of rabbit pox showing numerous papular lesions and a wide discolored zone of edematous infiltration. The variation in the size of individual lesions and the concentration of lesions in vascular areas as shown in this photograph were typical findings. Natural size.
- Fig. 2. Undersurface of the skin from a fatal case of infection showing discrete lesions of the hemorrhagic type and their relationship to blood vessels.  $\times 3/4$ .
- Fig. 3. Hind quarters of a young animal killed late in the course of disease. Areas of hemorrhage and mononuclear infiltration were widespread throughout the skeletal musculature. Natural size.

### PLATE 23

- Fig. 4. Lungs from a fatal case of infection in a young animal showing numerous subpleural miliary nodules. Similar lesions were found deep in the substance of the lung but pneumonic consolidation was absent. Natural size.
- Fig. 5. Liver and spleen from a young animal. Small focal areas of necrosis are distributed profusely throughout the liver. The spleen is enlarged and shows a few scattered discrete lesions. Microscopically, the sinuses were distended with large degenerating mononuclear cells and Malpighian corpuscles were poorly defined and consisted of similar elements interspersed with nuclear detritus. Natural size.
- Fig. 6. Spleen from an adult animal showing marked enlargement and large subcapsular foci of necrosis. Lesions of this kind were unusual. Natural size.
- Fig. 7. Lungs from an adult animal showing diffuse consolidation and large subpleural nodules with umbilicated centers. Compare with Figs. 8 and 9. Natural size.
- Figs. 8 and 9. Tongue. Large umbilicated nodules were commonly found in this situation. Microscopically, they consisted of subepithelial mononuclear infiltration which extended deeply into the underlying muscle, but the epidermis itself showed no specific changes. Natural size.

### PLATE 24

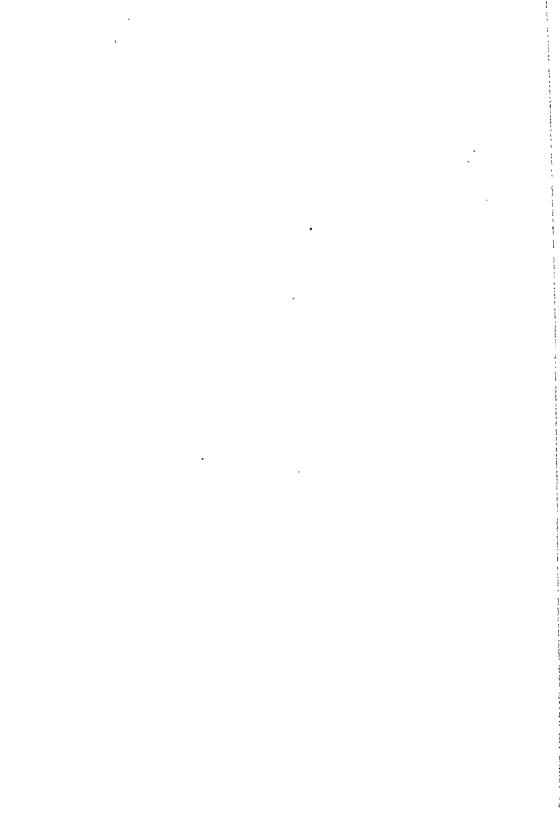
- Fig. 10. Section of the liver from an animal killed during the course of the disease showing degeneration of parenchymatous cells and an early focus of mononuclear cell infiltration in the portal connective tissue. In other sections all stages in the further development of this lesion could be traced from a more pronounced infiltration involving neighboring lobules to final necrosis. ×275.
  - Fig. 11. Section of bone marrow from a fatal case of infection in an adult

animal. There is widespread necrosis of hematopoietic tissue and no intact cells can be found. ×75.

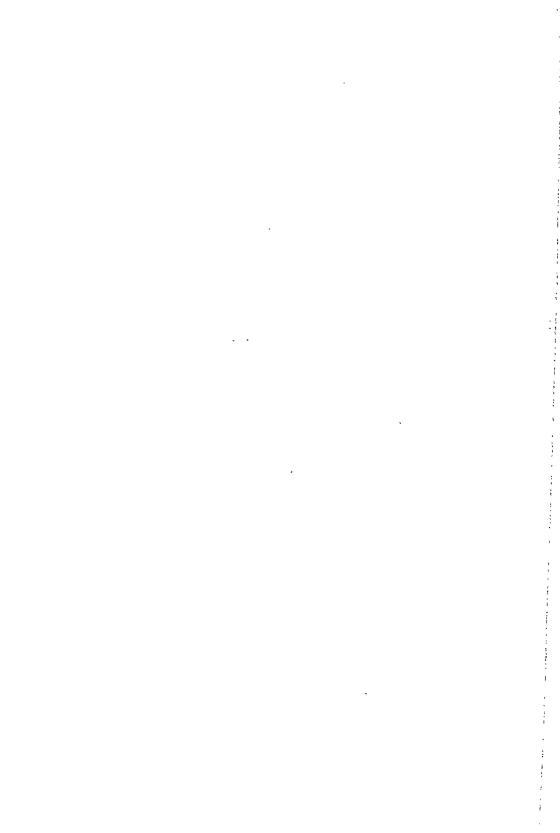
- Fig. 12. Section of lung from an animal killed in an early stage of disease. The lesion is deep in the substance of the organ and consists of a focal mononuclear infiltration with exudation into the alveoli and extension of the infiltration in alveolar walls toward the pleural surface. A blood vessel near the pleural surface contains an excessive number of mononuclear cells. ×90.
- Fig. 13. Section of the lip showing a diffuse infiltration of deeper tissues including the muscle with mononuclear cells and fluid. ×130.
- Fig. 14. Section through an early lesion in the skin of the ear showing mononuclear infiltration about a small blood vessel in the corium, destruction of endothelial cells and hyaline changes in the vessel wall. There were no specific epithelial changes. ×130.
- Fig. 15. Section through a lesion in the lung of an animal killed when the first external sign of infection was observed. The lesion has progressed to necrosis but has remained localized about two adjacent blood vessels which show advanced changes in the endothelial and muscular walls. ×75.













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# ON THE EXISTENCE OF A FACTOR INCREASING TISSUE PERMEABILITY IN ORGANS OTHER THAN TESTICLE

By ALBERT CLAUDE, M.D., AND F. DURAN-REYNALS, M.D. (From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, July 5, 1934)

In an earlier publication a report was made on an infection-enhancing effect obtained with testicle extract. This property is shared by certain other organ extracts but to a far less degree (1). As the enhancing power of testicle extract was later found to be associated with a modification of the skin permeability (2), extracts from various organs have been tested to determine their relative ability to cause this.

The dissociation between *spreading* and *enhancing* action (3) observed with certain tumor extracts, and the necessity of ascertaining where the spreading factor exists in the body, led us to undertake a systematic study of extracts from various tissues by following the spread of India ink particles in the rabbit skin. Such an investigation was also considered as an indispensable preliminary to the study of the physiology of the factor or factors, and of the mechanism of its action.

### Method

Adult rabbits of both sexes were used. The extracts were prepared by grinding the freshly removed organs with sand and 1, 1.5, and 2 volumes of Ringer's solution or water. The suspensions were centrifuged and 0.5 cc. of the cloudy supernatant fluid injected intradermally in the flanks of rabbits together with 0.25 cc. of Higgins' India ink, diluted 1:2. A similar control injection of 0.5 cc. of Ringer's solution or water plus 0.25 cc. of the ink was always made in the same animal. The spread of the ink was measured 1 and 24 hours after injection. In some cases precipitation of the tissue extract with 4 volumes of acetone, and re-extraction of the residue with variable amounts of water was resorted to with the hope of purifying and concentrating the factor. The technique, which had been very effective in the case of testicle extract, did not give better results than the use of fresh extract.

### EXPERIMENTAL

About 120 tests were carried out, each test involving the extract of an entire organ, or in the case of some extracts, of the pooled organs of 16 different individuals (rats). The average results with testicle extract were included for comparison. The findings are summarized

TABLE I
Occurrence of the Spreading Factor in Various Mammalian Organs

Organ extract tested	No. of tests	Average spreading of 0.5 cc. organ extract plus 0.25 cc. of India ink dilution	Average spreading of 0.5 cc. saline plus 0.25 cc. India ink dilution (control)	Ratio of active spread to spread of control	Positive cases with increased diffusion
		sq. cm.	sq. cm.		per cent
Rabbit lung	7	27.3	7.2	3.8	100
" spleen	7	18.1	7.2	2.5	100
" liver	7	8.5	7.1	1.2	42
" kidney	10	17.6	7.2	2.4	80
" ovary	2	20.4	4.1	5.0	100
" placenta	2	22.0	9.0	2.4	100
" brain	10	10.4	5.3	1.9	90
" skin	4	10.1	6.5	1.5	100
" blood serum	15	9.8	10.9	0.9	0
" striated muscle	3	3.2	5.4	0.6	0
" testicle	9	55.6	5.8	9.6	100
Rat lung	2	20.3	7.8	2.6	100
" spleen	5	24.2	7.4	3.2	100
" liver	5	26.7	7.4	3.6	80
" kidney	5	18.6	7.4	2.5	100
" brain	4	14.5	6.6	2.2	100
" blood serum	2	7.8	7.8	1.0	0
" testicle	10	40.4	5.8	6.9	100
Guinea pig lung	2	17.0	6.1	2.7	100
" " splcen	2	11.0	5.0	2.2	100
" " liver	2	17.5	5.0	3.5	100
" " kidney	2	9.0	5.1	1.7	100
" " blood serum	1	6.8	6.8	1.0	0
Calf thymus	1	12.0	4.0	3.0	100
Human placenta	1	9.0	4.0	2.2	100
" serum	7	7.4	7.0	1.0	0

in Table I. They clearly demonstrate the existence of spreading factors in all organs studied, although in a proportion far inferior to that existing in testicle, as judged by the India ink spreads. Striated muscle extracts might constitute an exception to this rule. In one investigation they were found inactive. Sera proved inactive in every case.

The quantitative differences in the spreading factor or the extracts studied were further brought out by dilution. Whereas testicle extract still increased the spread of ink when diluted to 1:400 (the highest dilution studied) extracts of other organs were no longer active at 1:10, and only in the case of lung and spleen extracts was there observed some spread at 1:5 dilution.

Another difference between testicle and other organ extracts consisted in the lack of regularity with which the latter yielded a spreading factor, contrasted with the perfect consistency of the former in this respect. This is indicated in the last column of Table I and holds especially for liver. Lung and spleen extracts, although fluctuating in their yields, always exhibited a certain activity in increasing the spread of ink.

The spread induced by the various organ extracts was similar to that obtained with testicle extract, but took place much more slowly. Nevertheless, lung and spleen extracts caused in certain cases considerable spreading in the hour following injection. In general though, the amount of the ink distributed through the area in which spread occurred was much less marked than in the case of testicle extract. The areas of spread when testicle extract was used looked always blackened by the ink, while the skin modified by the extracts from other organs, as shown by the spread of ink particles through it, was regularly lighter. Another detail to be mentioned is that the skin thickening (probably due to edema) was greater in the case of the latter. Skin treated with testicle extract was not swollen. Some of these points are illustrated in Table II, in which the areas and the density of the spreads produced by extracts prepared with the organs of a single rabbit were recorded 1 and 24 hours after injection.

<sup>&</sup>lt;sup>1</sup> Spinelli (6) has also found a spreading power in thyroid extracts. Muscle extracts, taken as control, were inert in 8 of his 15 trials. Testicle extracts were always active.

Summarizing we can state that practically every one of the 12 organs studied contains in widely varying amounts factors increasing tissue permeability, as shown by the spread through it of India ink, and that testicle and epididymis, lung and spleen seem to be the most active.

The possible rôle played by the testicle in bringing about the presence of the spreading factor in the other organs has been investigated. Their property of increasing tissue permeability was found to be retained after castration. The results obtained with rats' organs

TABLE II

Progression of the Spread with Various Organ Extracts

	Readings	Readings after 1 hr.		Readings after 24 hrs.	
	Area of spread	Density of spread	Area of spread	Density of spread	
	sq. cm.		sq. cm.		
Ringer's solution plus India ink (control)	. 4.1	±	4.2	±	
Spleen extract		1+++	29.0	1 +++	
Lung "	. 9.0	+	36.9	+++	
Kidney "	. 8.1	1+++	17.6	+++	
Brain "	. 6.2	++	10.5	++	
Liver "	. 8.4	++	8.0	++	
Muscle "	. 4.4	+	4.9	+	
Testicle " (average of 9 tests)	. 19.4	1++++	55.6	++++	

tested 5 weeks after castration have been included in the table. This observation indicates that the spreading factor is a usual constituent of these organs.

### COMMENT

The results of the present investigation clearly demonstrate the existence in nearly every organ tested of a factor active in increasing tissue permeability. It was shown in previous work that the same organ extracts possess the power of enhancing bacterial and virus lesions (1). In the case of extracts from testicle (2) and from invasive bacteria (3) a direct correlation was found to exist between the degree of spreading and the intensity of enhancing power. These observations make it probable that the same correlation may exist in

most of the organ extracts studied. Further experiments are necessary to test this assumption. It is sustained by the fact that certain azo compounds which are active spreading agents also enhance and increase the size of infectious lesions (4). The fact deserves mention, however, that spleen extracts not only failed to enhance vaccinal or staphylococcus lesions, but sometimes exhibited a definite inhibiting action. An analogous dissociation of the properties of organ extracts regarding the power to increase tissue permeability or to enhance infections has been met with in the case of certain melanomas (5). Likewise, extracts of some transplantable sarcomatous growths failed to enhance infectious lesions, although rich in spreading factor. such cases there was an indication that an anti-infectious agent was present in the tissue extracts, in addition to the spreading factor.2 For this reason we consider the permeability test more reliable for the detection of the factor in organ extracts than the original method of the production of lesions. Blood serum is completely devoid of both enhancing and spreading power.

Work is under way on the chemical relationship between the testicle factor and the factor obtained from other organs. The possibility of quantitative or qualitative variations at different periods in the life cycle is also being considered.

### SUMMARY

Many of the organs from animals of both sexes, including the ovary, have been found to contain in various proportion a factor or factors increasing tissue permeability. The potency exhibited by such active extracts was always less than that of extracts from testicle. Blood serum was found to be devoid of any spreading property.

In support of this view we can adduce the fact that, while extracts prepared from testicle immune to vaccine virus still retain to the same extent as normal testicle the power of increasing dermal permeability, they fail to enhance the virus lesions and indeed actually suppress them. The immune testicle extract may have spread the vaccine suspension over a large area, but it has spread an inactivated material. It is worth noticing in this connection that Felton (7) has increased the virulence of pneumococcus by repeated automatic transfers in lung medium and decreased it by similar transfers in spleen medium.

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# THE ACCUMULATION OF IRON IN TUBERCULOUS AREAS

# IV. THE EFFECT OF FERRIC CHLORIDE ON THE COURSE OF TUBER-CULOSIS IN REINFECTED RABBITS\*,†

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PLATES 25 TO 27

(Received for publication, July 10, 1934)

Previous studies by the writer have demonstrated that repeated intravenous injections of dilute ferric chloride solution are followed by an accumulation of iron in tubercles. Concomitantly with this accumulation the course of the disease in rabbits was found to be retarded, as evidenced by an increase in the survival time and by the less extensive involvement of the lungs of experimental animals as compared with controls that had had the disease for the same length of time (1-4). These results were obtained in two independent series of experiments. In the earlier investigations bovine tuberculosis was induced by intravenous inoculation of a virulent culture. When the intravenous ferric chloride treatment promptly followed subcutaneous injection of the bacilli, the dissemination of the latter from the site of inoculation was retarded (5). In a preliminary communication (6) it was shown that reinfected rabbits receiving repeated ferric chloride injections survived for more than 6 months after the death of the last control, which succumbed about 4 months following reinfec-

The present experiments give in greater detail some of the data on the combined effect of partial immunity and ferric chloride administration on the course of experimental tuberculosis. A comparison is

<sup>\*</sup> Aided by grants from the DeLamar Mobile Research Fund, and from the Committee on Therapeutic Research, American Medical Association.

<sup>†</sup> Read before the American Society for Experimental Pathology, New York, March 29, 1934. 443

made with the results of treatment of a non-vaccinated group of tuberculous animals. Some observations are brought forward indicating the mechanism possibly responsible for the favorable effect of ferric chloride in experimental tuberculosis.

TABLE I

Effect of Ferric Chloride Injections on Tuberculous Rabbits

	Control animals			Experimental animals			
	Rabbit No.	Weight at beginning of experi- ment	Survival time after inoculation with virulent tubercle bacilli	Rabbit No.	Weight at beginning of experi- ment	Amount of 0,25 per cent ferric chloride injected	Survival time after inoculation with virulent tubercle bacilli
	}	gm.	days		gm.	cc.	days
Group I, non-vac-	7-71	2230	91	7-60*	2015	201	112
cinated	7-31	2195	102	7-51	1820	219	161
	7-80	1670	126	7-59	1860	214	287
	7-83	3010	162	7-30	2200	218	326
	7-81	2635	170	7-79	1695	202	344†
Average		130				246	
Increase	in surviv	al time of	experime	ntal anim	als: 89 pe	r cent	
Group II, prelimi-	5-97	2900	47	6-07*	2450	232	81
nary vaccination	6-26	2060	55	5-41*	2500	278	110
with relatively	6-02	2250	67	6-16	2125	278	131
avirulent strain	6-24	2520	108	5-96	2200	278	334†
{	6-28	2500	130	6-04	2955	278	334†
Average			81				

Increase in survival time of experimental animals: 144 per cent

### EXPERIMENTAL

The technic followed was practically the same as that employed in the carlier experiments; for this reason it will not be taken up in great detail.

In brief, a series of 10 Flemish brindle rabbits was inoculated subcutaneously in the thigh each with 0.05 mg. of a virulent bovine Ravenel strain of tubercle bacilli. Intravenous injections of ferric chloride (0.25 per cent in distilled water) were started immediately in 5 of these rabbits and continued for a period of about

<sup>\*</sup> Superimposed upper respiratory infection at time of death.

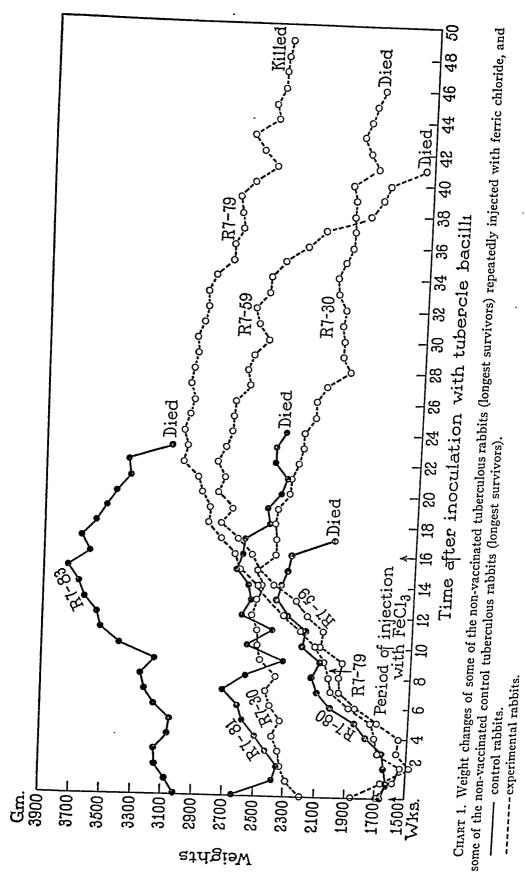
<sup>†</sup> These animals were killed; all others died of their diseases.

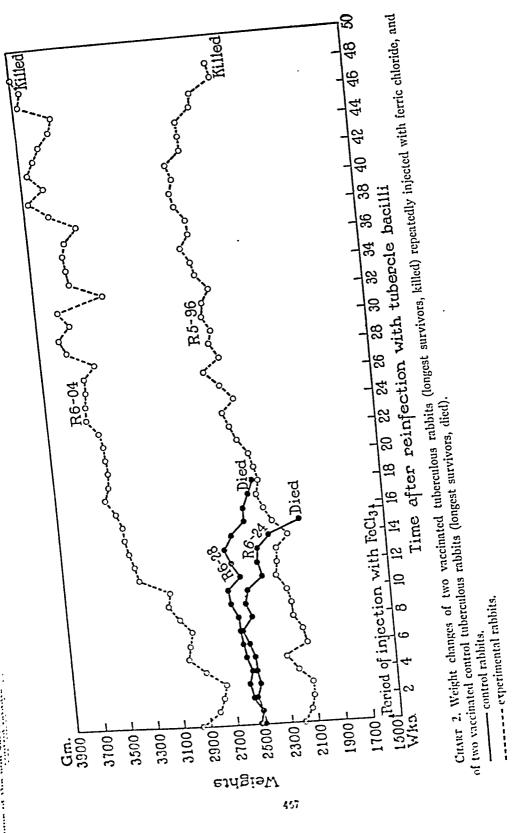
4 months. As a rule about three injections of 6 cc. each of the iron salt were made every week. At the beginning the injections were performed more frequently and the individual doses were larger (10 cc.). Subsequent observations indicated that 5 to 6 cc. of 0.25 per cent ferric chloride (lukewarm) was innocuous to rabbits when injected slowly into the circulation. The stock solution of ferric chloride was kept on ice when not in use.

A second group of 10 brindle rabbits was inoculated intravenously each with 0.005 mg. of a relatively avirulent strain of bovine tubercle bacilli (Cernay). About 6 weeks later each of these animals received subcutaneously in the thigh 0.05 mg. of a virulent bovine Ravenel strain of tubercle bacilli precisely as in the first series of rabbits. The injections of ferric chloride were started promptly in 5 of the group and were continued for about 4 months as described above. The purpose of this second series of experiments was to determine whether partial immunity induced by primary inoculation would enhance the retarding effect of ferric chloride.

### Effects of Ferric Chloride on Survival Time

The results of the experiments are shown in Table I. Group I summarizes the observations on the non-vaccinated rabbits. Five controls lived from 91 to 170 days with an average survival time of 130 days. Four of the experimental rabbits survived from 112 to 326 days and one was still alive 344 days after subcutaneous inoculation with virulent tubercle bacilli. At that time this animal was sacrificed and a thorough postmortem examination was performed. When this last animal is included in the calculations, the average longevity of the experimental rabbits is found to be 246 days, an increase of 89 per cent over the survival time of the controls. The weight changes of the longest survivors of the control and experimental animals in this group are plotted in Chart 1. It is clear that death follows shortly after the controls begin to lose in weight (see control Rabbits 7-83, 7-S1, and 7-S0, Chart 1). On the other hand the weight curves of the experimental rabbits show a definite protracted course even when the animals start to show a loss in weight (see experimental Rabbits 7-59, 7-30, and 7-79, Chart 1). The controls began losing weight between the 12th and the 15th week of their disease; while with the exception of Rabbit 7-30, the experimental rabbits began to show a decline in their weight curves at the 22nd week of their disease; i.e., about 6 weeks after discontinuing the ferric chloride injections. In brief, the data both on the increase in survival time and on weight





changes with administration of ferric chloride are in complete accord with the writer's previous observations (3, 4). The more marked increase in longevity obtained in this group of animals as compared with the previous series is presumably referable to the effect of ferric chloride on the milder type of disease induced by subcutaneous inoculation of tubercle bacilli. In the earlier experiments the disease had been induced by intravenous inoculation of the microorganisms.

Group II in Table I summarizes the results obtained in the reinfected series of rabbits. These animals were not inoculated with the virulent bacilli (Ravenel) simultaneously with the non-vaccinated rabbits of Group I; nor was the same suspension employed. Hence the two series can be compared only in regard to the respective differences between survival time of experimental and control rabbits. However, the actual values in survival time of Group I are not comparable with those of Group II. In the latter the 5 controls lived between 47 and 130 days with an average survival time of 81 days. experimental rabbits succumbed 81, 110, and 131 days after subcutaneous reinoculation with virulent tubercle bacilli. Two of the animals had superimposed upper respiratory infections at the time of death. The 2 remaining experimental rabbits (Nos. 6-04 and 5-96) progressively increased in weight, as indicated in Chart 2. lived for over 6 months after the death of the last control animal. On the 334th day after reinoculation with virulent tubercle bacilli these 2 animals were killed and thorough postmortem examinations were performed. The average survival time of the experimental group is unknown, since 2 of the animals were finally sacrificed. However, prior to the autopsy of the 2 survivors amongst the experimental animals the average life span of this group of rabbits was 198 days as compared with 81 days in the control series, or an increase of 144 per cent over the average survival time of the controls.

In Chart 2 are plotted the fluctuations in weight of the longest survivors of both control and experimental reinfected rabbits. Here again control Rabbits 6-28 and 6-24 succumbed shortly after they began losing weight; whereas experimental Rabbits 6-04 and 5-96 maintained steady weights for over 200 days after the death of the last of the controls. Small temporary losses in weight, as indicated on the chart, were associated as a rule with summer heat waves.

With relief in weather conditions, the animals invariably regained their original weights. The effects obtained by repeated intravenous injections of ferric chloride in reinfected tuberculous rabbits are evidently even more striking than those in the non-vaccinated animals of Group I (Table I). This doubtless is referable to the partial immunity induced by primary inoculation which results in a less acute form of tuberculosis upon reinfection with a virulent strain. The degree of immunity conferred by primary inoculation is associated with considerable individual variation. The immunity may be of sufficient magnitude to induce a high degree of resistance in control reinfected animals; i.e., those receiving no iron. In a recent study on the BCG and human strains of tubercle bacilli, the writer has found this to be the case. When, however, the Cernay strain of bacilli was employed for primary inoculation, as described in the foregoing experiments, only a relatively mild degree of immunity must have been induced, since all the controls succumbed with considerable tuberculous involvement.

## Effect of Ferric Chloride on the Extent of Tuberculous Lesions

The morphology of the organs in tuberculous rabbits treated with ferric chloride has already been described (3-6) and will be touched upon but briefly.

As a rule, gross lesions were seen only in the lungs, the kidneys, and at the site of subcutaneous inoculation. In the latter, the lesion appeared in the form of a fairly well circumscribed sacculated abscess which frequently buried itself deep into the anterior muscles of the thigh. It was filled either with a yellowish, suppurating material, of a fairly fluid consistency; or else contained a more viscous substance which formed a partially inspissated caseous mass. This local subcutaneous lesion seemed (in some cases) to be slightly larger in controls than in experimental animals. The kidneys presented the usual picture of discrete caseous foci, situated primarily in the cortical region. The gastrointestinal tract was infrequently involved in the form of miliary tubercles located chiefly in the fleum and in the neighborhood of the cecum. Though the appearance of the lungs varied somewhat in both series, their constant involvement influenced their selection as the main object for study, in comparing lesions of control and experimental rabbits. In the control animals of the non-reinfected group (I), the lungs at post mortem revealed extensive confluent pulmonary tuberculosis only in the longest survivors (e.c., Rabbits 7-83 and 7-81, Table I). The animals which succumbed earlier presented relatively fewer discrete lesions. The reason for this well known variation in extent of involvement in regard to time of death is not clear. The lungs of experimental rabbits of this group were often characterized by an intense congestion of the parenchyma interspersed by caseous foci; a tendency to confluence was noted at the bases and margins of the organ.

The extent of involvement showed some degree of variation, but it is noteworthy that in the longest survivors of the experimental group the degree of tuberculosis was far less than in some of the longest surviving controls. (See Fig. 1 and Table I, Rabbit 7-83, control, and Rabbit 7-30, experimental.) Some of these experimental rabbits were still alive 5 to 6 months after the death of the last of the controls (Table I, Group I), and yet the extent of tuberculous involvement in the former was considerably less than in the latter.

There is another point of difference between experimental and control rabbits which may be mentioned here. In animals treated with ferric chloride, the presence of iron is demonstrated (by the Prussian blue reaction) not only in caseous areas of lung tissue, but also within mononuclear phagocytes. The iron appears in the form of yellowish brown pigment, indistinguishable from hemosiderin, and is practically always observed in abundance within the sinuses and reticular cords of the spleen; in the reticular cells of the bone marrow; and fairly frequently in the Kupffer cells of the liver. In the latter organ, however, yellow, iron-containing granules were also occasionally found within the parenchymatous cells of animals treated with the iron salt. While it is true that such pigmented material is occasionally seen in the spleen of a normal rabbit, it has not been encountered by the writer, at least in the present series of experiments, either in the bone marrow or Kupffer cells of the liver of untreated animals, although Rous and others have observed normal rabbit livers containing red cells in various stages of degradation together with hemosiderin. The present observations indicate that some degree of hemosiderosis was evidently induced in the experimental animals by repeated intravenous injections of ferric chloride. The injections had no evident effect on the hemoglobin percentage. A slight increase in the reticulocyte level when compared with that of controls was noted. The average reticulocyte count in the experimental animals studied was 1.2 per cent, while that of the controls was 0.8 per cent. This is doubtless due to some degree of hemolysis in the ear

vein occasioned by the sudden introduction of a relatively large volume of hypotonic ferric chloride (5 to 6 cc. of 0.25 per cent concentration). It is doubtful, however, whether this problematic, slight hemolysis is sufficient to account for the extensive hemosiderin deposition in the various organs. When a 0.25 per cent ferric chloride solution rendered isotonic with mammalian blood (by the addition of 680 mg. of sodium chloride to 100 cc. of solution) was employed instead of the hypotonic solution, the same picture of hemosiderosis was observed. Recent studies by the writer have shown that the addition of 0.25 per cent ferric chloride to tissue cultures of mesenteric lymph nodes is followed by the appearance in large mononuclear cells of iron-containing pigmented material indistinguishable from hemosiderin. The above observations which are being reported in greater detail in separate communications (7) indicate that hemosiderin is not derived solely from the product of hemoglobin degradation, but may result from the action of cells in general upon iron contained in body fluids. For this reason it has been suggested that, for the pigment in question, cytosiderin would represent a more accurate name than the more specific term hemosiderin. Studies are now in progress to determine whether this iron-containing material plays any rôle in protracting the course of the disease.

In regard to the degree of tuberculosis in the control and experimental reinfected rabbits of Group II, the general involvement was somewhat similar to that described above for the non-vaccinated series. The lungs of controls showed in practically all cases widespread confluent caseating tuberculosis, a picture differing markedly from that observed in animals treated with ferric chloride. Those members of the latter group that succumbed during the period of ferric chloride injection (Rabbits 6-07, 5-41, and 6-16) frequently exhibited extensive pulmonary congestion. In these congested lungs a moderate number of discrete caseous foci were scattered about. In the 2 rabbits (Nos. 5-96 and 6-04. Table I) which survived for more than 6 months after the death of the last control, the amount of vascular congestion was not appreciable. One may recall that at the time that these animals were sacrificed, the ferric chloride injections had been discontinued for about 32 weeks. Hence it is quite reasonable to suppose that the congestion in the lungs is probably associated with the accompanying

intravenous injections of the ferric salt. The postmortem findings in these 2 rabbits, which were killed 334 days after reinoculation with virulent tubercle bacilli, have been adequately described already in a short preliminary note (6). Suffice it to point out here that the scant tuberculous involvement in these 2 animals was noteworthy, especially when compared to the massive, confluent caseating tuberculosis in the lungs of the longest control survivors which had died more than 200 days previously (see Fig. 2). Connective tissue proliferation frequently occupied a more prominent position at the peripheries of caseous foci in experimental rabbits than in the controls. (8) has pointed out that the bovine tuberculous lesion in the rabbit is characterized by a slight degree of fibrosis. This is well exemplified in the lungs of the longest surviving control (No. 6-28, Fig. 4) where at the peripheries of large caseous areas, connective tissue cells are few in number. It is interesting to note, on the other hand, in experimental Rabbit 6-04, which was still alive 334 days after reinoculation with tubercle bacilli, that the few large caseous areas present in the lung are circumscribed by a fairly wide zone of connective tissue proliferation (Fig. 5). Fibrosis in the tubercle, as Opie recently pointed out, is doubtless an expression of resistance to the further spread of the disease (9).

The mechanism to account for the favorable effect of ferric chloride is now under investigation. For the present we shall only call attention to the frequent finding of intense congestion in the pulmonary tissue of animals repeatedly injected with the iron salt, particularly if these animals are killed within a relatively short time after the period of ferric chloride treatment. The reader is referred to Figs. 1 and 3 for illustrations of the degree of congestion in the lungs of experimental rabbits. The frequent occurrence in the lungs of treated animals of a zone of engorged capillaries about tubercles, at times associated with some fibroblastic proliferation, suggests the end-stage of a superimposed inflammatory reaction induced by the accumulation of iron in tubercles (Fig. 6). This possibility is supported by the fact that ferric chloride, in the concentration employed, is in itself an intense inflammatory irritant. This is quite evident when the salt is injected into the dermis of an animal. The lungs of animals surviving long after cessation of treatment were often characterized

by moderate connective tissue proliferation at the peripheries of tubercles (Fig. 5, Rabbit 6-04). This fibrous zone, in its location with respect to the tubercles, corresponded in a general way to the area of engorgement found at an earlier stage. The presence of areas of congestion in control tuberculous rabbits was observed in a relatively small percentage of cases. In support of the concept that ferric chloride is responsible for the reactive pulmonary changes the following histological data were collected. The lungs of 24 controls were studied for the presence of vascular congestion and connective tissue proliferation about caseous areas. Congestion was found in 10 cases or 42 per cent of the lungs studied; while the incidence of fibrous tissue was noted in 12 cases; i.e., in 50 per cent of the organs examined. The lungs of 27 experimental rabbits that had received varying amounts of ferric chloride yielded the following information: 16 of the lungs or 60 per cent of cases revealed marked vascular congestion: while 24 lungs or 90 per cent of the total cases were characterized by varying degrees of fibrosis.

In connection with these facts, there are in the literature some interesting clinical and pathological observations which may be mentioned. About 90 years ago, Rokitansky pointed out the rarity of pulmonary tuberculosis in lungs that were passively congested. He even asserted that cardiocirculatory diseases producing passive congestion of the lungs were a preventative of phthisis (10). Weiss (11) expressed the opinion that in mitral stenosis accompanied by pulmonary congestion tuberculosis of the lungs was infrequent. More recently Hyndman and Landt (12) in some experimental studies came to the conclusion that the number of tuberculous lesions in congested organs was smaller than in the corresponding non-congested organs of control animals.

#### DISCUSSION

The results of the present series of experiments show definitely that repeated intravenous injections of dilute ferric chloride solution retard markedly the course of development of tuberculosis in rabbits. Furthermore, the present observations indicate that the combined action of partial immunity induced by a first infection and of ferric chloride administration enhances the favorable effects of the iron salt in reinfected animals. The implications of these findings are obvious, especially when it is recalled that the tuberculosis of white adults

is generally regarded as a disease of reinfection following the primary infection of infancy or childhood.

The results of seven independent series of experiments representing a total of 100 rabbits are summarized in Table II. The studies on

TABLE II
Summary of All Studies on Effect of Ferric Chloride in Tuberculous Rabbits

Series	Results	Total No. of	Average sur	Increase in survival time	
	published in	rabbits	Control animals	Experimental animals	of experimental animals
			days	days	per cent
*1	1932	16	61	109	78
*2	1933	20	94	135	44
3	1934	10	130	246	89
†4	1934	10	81	198	144
Average			91.5	172	89
Total No. of rabbits		56			

<sup>\*</sup> Series 5 (1933). 16 tuberculous rabbits sacrificed between 45th and 79th day of the disease. Extent of tuberculous involvement in lungs of experimental animals considerably less than in controls

Total No. of rabbits in all series: 100

survival time include 56 rabbits. The average survival time of all control rabbits is 91.5 days; that of the experimental animals is 172 days. The average increase in the survival time of experimental rabbits is 89 per cent. Series 5 and 6, comprising 35 animals, represent

Series 6 (1933). 19 tuberculous rabbits sacrificed between 35th and 108th day of the disease. Tubercle bacilli inoculated subcutaneously. Experimental rabbits showed definite retardation in spread of the bacilli as evidenced by extent of lesion, particularly in the lungs

<sup>\*</sup> Series 7 (1931). 9 rabbits with bovine tuberculosis; 2 of these treated for about a month with ferric chloride intravenously prior to inoculating with tubercle bacilli. They survived considerably longer than controls: 124 and 115 days respectively. Average survival of 7 controls: 54 days

<sup>\*</sup> Bovine tuberculosis induced by intravenous route; all others were inoculated subcutaneously.

<sup>†</sup> This group received a preliminary vaccination with a relatively avirulent strain of tubercle bacilli (Cernay).

a study of the extent of lesions in control and experimental animals that had the disease for the same length of time. These, as previously reported (4, 5), were in entire accord with the results obtained on survival time; a comparison of the pathological lesions substantiated the fact that the course of the disease following ferric chloride administration was protracted.

The explanation for this favorable action on the part of ferric chloride is still somewhat problematical owing to the multiplicity of factors concerned. It is possible that the retarding effect on the development of the disease may be the resultant of several factors. The frequent appearance of intense vascular congestion associated with some fibroblastic proliferation in tuberculous lesions of iron-treated animals suggests the end-stage of a superimposed inflammatory reaction induced by the accumulation of the iron in tubercles. The introduction of this additional synergistic element would enhance the various factors in the tubercle which naturally tend to dispose of the bacilli. Furthermore the frequent deposition within mononuclear phagocytes of an iron-containing pigment, indistinguishable from hemosiderin, may, by activating the cells of the reticulo-endothelial system, constitute a factor in the favorable effect of ferric chloride administration. The recent observation of Rous and Beard (13) that Kupffer cells loaded with ferromagnetic iron oxide can be dislodged with relative ease into the circulating blood from their attachment to the liver parenchyma renders it conceivable that cells of the reticuloendothelial system loaded with the iron-containing pigment may be more readily detached and may thus be disseminated in greater numbers to tuberculous foci.

### SUMMARY AND CONCLUSIONS

Repeated intravenous injections of dilute ferric chloride solution in tuberculous rabbits markedly retard the development of the disease as evidenced both by a prolongation in the survival time and by comparison of lesions in control and experimental animals.

Partial immunity induced by first infection combined with ferric chloride administration enhances in reinfected animals even more strikingly the favorable effects of the iron salt. Some of the experimental animals were still alive and apparently well about 6 months

after the death of the last of the controls which succumbed about 4 months after reinfection with virulent tubercle bacilli.

Factors that may perhaps account for the favorable effect of ferric chloride in experimental tuberculosis have been discussed.

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### EXPLANATION OF PLATES

### PLATE 25

- Fig. 1. Comparison of lung of (a) Rabbit 7-83 (control, 162 days, non-vaccinated group) and that of (b) No. 7-30 (experimental, 326 days, non-vaccinated group). Both of these animals were allowed to die of the disease. Rabbit 7-30 had received by repeated intravenous injections a total of 218 cc. of 0.25 per cent ferric chloride. Even though the experimental animal survived over 5 months after the death of the control, the extent of tuberculous involvement in its lungs is distinctly less than in the control animal. The congestion in the lungs of the experimental rabbit is pronounced.
- Fig. 2. Comparison of lungs of (a) Rabbit 6-28 and (b) Rabbit 6-24 (longest control survivors, 130 and 108 days respectively, vaccinated group) and those of (c) Rabbit 5-96 and (d) No. 6-04 (longest experimental survivors, killed on 334th day after reinfection). Each experimental rabbit had received a total of 278 cc. of 0.25 per cent ferric chloride intravenously. They were killed more than 6 months after the death of the last of the controls. The amount of tuberculous involvement is scant in comparison with the extensive confluence of lesions in the controls.
- Fig. 3. Comparison of lung of (a) Rabbit 8-21 (control, killed on 63rd day of disease) and that of (b) No. 8-15 (experimental, killed on 63rd day of disease). This

rabbit had received a total of 57 cc. of ferric chloride. The disease was induced by intravenous inoculation of the bacilli. The involvement in the control lung is extensive as compared to that of the experimental animal. The intense congestion in the lung of the iron-treated rabbit is striking. About 100 more viable bacilli were cultured and recovered as individual colonies from the control than from the experimental lung.

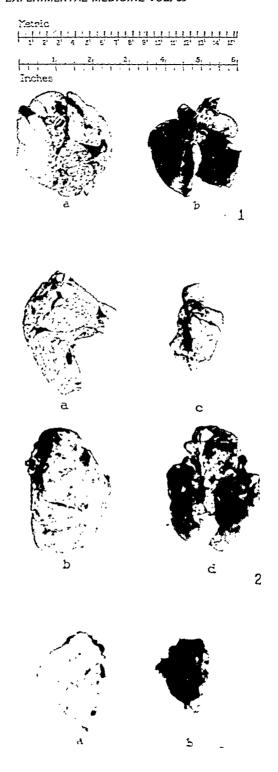
### PLATE 26

- Fig. 4. Photomicrograph of lung section of control Rabbit 6-28 (died 130 days after reinfection) showing extensive caseation with diffuse cellular infiltration at periphery. There is practically no evidence of any fibrosis. × 162.
- Fig. 5. Photomicrograph of lung section of experimental Rabbit 6-04 (killed 334 days after reinfection) showing at periphery of large area of caseation a moderately dense zone of connective tissue proliferation. Compare with Fig. 4 (control Rabbit 6-28). × 85.

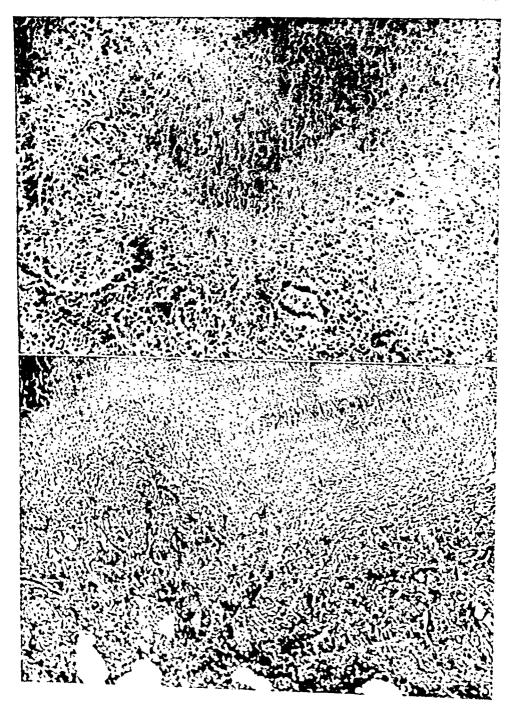
### PLATE 27

Fig. 6. Drawing of microscopic section through lung of an experimental rabbit that had received intravenously a total of 226 cc. of 0.25 per cent ferric chloride administered over a period of about 10 weeks. Note the dilatation, moderate engorgement, and large number of small vessels at the periphery of a caseous area. There is a slight connective tissue reaction. × about 500.



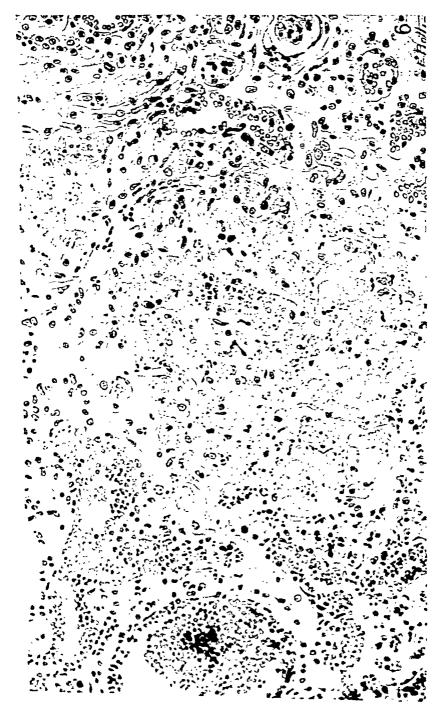






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## THE PARTICIPATION OF SKIN LYMPHATICS IN RI OF THE LESIONS DUE TO INCISIONS AND BUR

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PLATES 28 TO 30

(Received for publication, June 10, 1934)

The lymphatics of human skin participate in the responses slight injury. As our previous work has shown (1), poorly divital dyes, intradermally injected, enter the superficial lyacapillaries of the skin, and ordinarily are retained by them for minutes before escape occurs into the interstitial tissue. Consincreases in the permeability of the walls of these vessels, a fested by an almost immediate escape of dye from the confollow such stimuli as the application of gentle heat, ultraveradiation, or a firm stroke over the surface, too slight to about skin. Studies of the lymphatic capillaries in the ear of the have disclosed similar changes in the permeability of the these vessels (2, 3). Here too a scratch insufficient to break a light stroke with a blunt instrument, exposure to ordinary or an increase in temperature to but 5° above body heat stinduce a great transient increase in the permeability of the ly

The changes described have been effected without causi injury. In the present paper we wish to report upon the all in the permeability of the walls of lymphatic capillaries of the ear in and about regions subjected to burns or incision. Our has been to examine the responses of lymphatic capillaris frankly pathological conditions, with special reference to the ing of these channels during the formation and resolution inflammatory processes.

#### Methods

We have found the car of the mouse an ideal structure in which directly the changes in lymphatic permeability as they occur in liv

As in the preceding work (3), mice of 16 to 18 gm. body weight were best suited to our purpose. In all of the experiments the animals' ears were observed at various intervals after injury had been produced under an anesthetic. When the interval was many hours or several days, ether anesthesia was used when the injury was produced and the final observations were made under complete sodium luminal narcosis. When the times of injury and final observation fell within a period of only a few hours, one anesthetization with sodium luminal sufficed for both. As in the earlier work, 0.125 cc. of a 2 per cent aqueous solution of freshly dissolved sodium luminal was given subcutaneously for each 10 gm, of body weight. All examinations were made under the binocular microscope by a method already described (2).

To render the lymphatic capillaries visible and to test for alterations in the permeability of the lymphatic wall, a highly indiffusible vital dye, pontamine sky blue (2, 3), was injected locally into the skin of the upper surface of the outer edge of the ear. It enters directly the lymphatics draining the region through rents caused by the injecting needle, and extends along the local network rendering it clearly visible (3). In all the experiments save where specific mention has been made, 0.1 cc. of fresh aqueous isotonic pontamine sky blue (21.6 per cent) was added to 2 cc. of a mixture of 1 part mouse serum and 3 parts Tyrode's solution, yielding a final mixture of approximately 1 per cent of dye in an isotonic menstruum of protein content similar to that reported for peripheral lymph (4-7). This was regularly used to obtain comparisons of the rates of dye escape from the lymphatics after different injuries or at varying intervals after a standard injury. It will be referred to as standard pontamine solution. When it entered the lymphatic channels of the normal ear, no perceptible dye escape took place from them for 10 to 15 minutes. In many of the present experiments, on the other hand, so rapid an escape of dye resulted in consequence of the injury to the lymphatics that we were led to employ a suspension of particulate matter to test whether or not there were openings in their walls. For this purpose dialyzed non-waterproof India ink (Higgins' American drawing ink), or else "Hydrokollag," was injected together with the dye solution. The method of preparation of these suspensions has already been described (2, 8). Further to compare changes in the permeability of the blood vessels of the injured ears with the changes occurring in the lymphatics, pontamine sky blue was injected intravenously into many of the mice. The dye was given as described in earlier papers (9-11), employing 0.05 cc. of a 21.6 per cent solution mixed with an equal volume of Tyrode's solution.

To study the physiology of the lymphatics during the progress and restoration of wounds or inflammatory processes, and to render our results comparable from animal to animal, it was necessary to devise methods of traumatization which could be standardized for repetition. This was accomplished in several ways to be described below. Heat was applied in such a manner that mild first, second, or third degree burns were obtained at will, the more severe burns resulting in necrosis.

## The Lymphatic Changes Resulting from Skin Incisions

The effects of cutting through the skin were first observed. The incisions were made under the binocular microscope, so that the depth and extent of the wound could be accurately controlled.

The part played by the lymphatics in the process of wound healing is not known. In descriptions of the phenomena of wound healing there is little if any mention of these vessels. How do they behave during inflammation? What is their share in the healing of a wound? Do they become blocked like the blood capillaries when they are cut across? When they reconstitute during the healing process are the lymphatics more permeable than normal or less so? These and many other questions suggest themselves.

In our experiments the lymphatics of the skin of the mouse's ear were rendered visible by local injections of standard pontamine solution, at various times after incising the upper surface of the ear superficially or deeply. In the majority of instances healing took place rapidly and the behavior of the lymphatics could be studied during the healing processes.

Mice were anesthetized with ether and under the binocular microscope cuts about 1 cm. long were made half way between the tip and the base of one or both cars. In some cases the incisions were very superficial, involving only the corium and the subpapillary layer at most, and in these no hemorrhage developed. Probably only the most superficial lymphatics were severed. In other instances incisions were made deeply enough to cut the larger veins and arteries, while in some the upper surface was cut through to the cartilagenous plate of the ear. In still other experiments the cut went completely through the ear. Save in the animals with superficial incisions, more or less hemorrhage ensued, varying from small capillary ecchymoses to frank bleeding of the largest vessels.

A considerable number of mice were operated upon at one time, and at half hour intervals for 7 hours afterwards, groups of three animals each were anesthetired with luminal and local and intravenous injections of dye were made to render the blood vessels and lymphatics visible. On subsequent days other groups of six to ten animals were examined in the same way until all signs of wound reaction had disappeared.

The behavior of the lymphatics after incision differs strikingly from that of the blood vessels in certain respects. Where cut across the lymphatic channels remain open for a variable period of time, often for 24 to 48 hours or more, instead of constricting at once and closing as do the blood vessels.

Many of the blood corpuscles escaping into the tissue during the hemorrhage after incision pass directly into the lymphatics that are laid open, and continue to do so for many hours. Large numbers of erythrocytes are often found in the lymphatics both distal and proximal to the cut, as late as the day after making the incision. In instances showing this the lymphatics about the cut stand out clearly by reason of the contained blood. Clotting has not occurred and with a micro-spatula the intralymphatic cells can be moved in either direction. In relation to this finding, Clark and Clark (12) in their studies with transparent chambers in the rabbit's ear, report that holes made in lymphatics at the time of operation remain open several days.

When standard dye solution was injected at the ear margin beyond a transverse cut on the same day that the cut was made the channels at the periphery of the ear filled as usual but then emptied their stained contents directly into the incision where they had been cut across. Fig. 1 shows a photograph of the ear of a mouse injected in the usual manner 5 hours after a transverse incision. The wound became filled with blue dye which flowed without hindrance from the severed lymphatics. In a few experiments, instead of injecting the incised ears a tiny crystal of dye was pushed, under the guidance of the microscope, into a minute intradermal puncture wound at the periphery of the ear. The regional lymphatics took up the dissolving dye and within 15 to 20 minutes colored fluid could be seen passing from the severed lymphatic capillaries into the incision. Constriction and spasm of the small blood vessels severed were so immediate and effective, on the other hand, that often no bleeding occurred.

In eight instances within half an hour to 5 hours after a superficial cut had been made, a drop of standard dye solution was placed on the incision. In 3 or 4 minutes colored fluid appeared in the lymphatics on both sides of the wound. Those distal to it lying between the incision and the ear tip filled for the distance of a single pear-shaped segment only, that is to say to the nearest valve; those proximal, extending from the incision to the base of the ear, drained away dye solution toward the head. The finding was not invariable but was frequent enough to prove conclusively that the lymphatics were still open. Whether or not they took up dye seemed to depend upon whether it gained access to them through the fibrin clot in the wound.

The local injections at the ear margin showed not only that the severed lymphatics had failed to close but that the lymphatic capillaries distal to the cut but severed by it were more permeable than usual. Dye began to escape through the walls of these channels within 2 or 3 minutes after it had entered them, and always many localized

dye ecchymoses were seen, as though the channels had received direct local injury, which was not the case (2). In the unharmed ear standard pontamine solution remains within the channels for 10 to 15 minutes before any visible escape takes place (3, 2).

In most instances in which local injections of dye were made at the outer ear margin after a transverse cut had been made, not only were those lymphatic channels filled which drained directly into the cut, but others which skirted it at either end. These intact vessels carried the dye as usual to the base of the ear but they also passed along some of it, by side branches, into the region directly proximal to the injury and even discharged some into the wound itself by collaterals connecting with the open lymphatics in its proximal margin. Like the channels distal to the incision, the proximal collaterals showed an increased permeability to standard pontamine solution, dye beginning to escape from them 2 or 3 minutes after it had entered. Both distally and proximally to the wound, therefore, the lymphatics were more permeable than elsewhere in the ear. Within 5 or 6 minutes after an injection the margins of the cut for a distance of 2 or 3 mm. were stained a bright, diffuse blue.

The results of intravenous injections of dye on the same day the cut was made furnished an enlightening contrast to these findings.

The animal gradually became blue all over save for an area of pallor in and about the incision for a distance of 2 to 3 mm. It was plain that occlusion of the severed blood vessels and spasm of those near-by prevented dye from escaping into the immediate region of injury. Just beyond the area of pallor, the blood vessels allowed more dye to escape than elsewhere in the ear, and within 5 minutes after an injection the pallid incision was surrounded by a ring of diffuse blue stain

Sixteen animals were examined under sodium luminal anesthesia on the day following incision.

These had received superficial cuts in one ear,—extending only through the subpapillary layer of the corium,—and deep cuts in the other ear, reaching to the cartilaginous plate and severing the overlying blood vessels. In all instances both cars were hyperemic and moderately edematous, with the edema most marked distal to the incision, though definite on the proximal side as well. In such regions the ears showed "pitting upon pressure" by a blunt instrument. They were thick, the skin tense, and when punctured by a sharp needle fluid escaped. The wound was ringed by dilated, tortuous capillaries.

After injecting standard pontamine solution intradermally at the ear margin on the day following incision the lymphatics draining the region filled as usual and carried colored fail I to a point about 2 mm, from the incision. In most instances

there was in this region a tendency for the lateral connecting channels to divert the dye to the extremities of the wound, and thence into main trunks leading around the incision toward the ear base. In four animals, though, dye solution also entered the wound showing that some of the severed lymphatic capillaries were still open. As a rule in each animal many dye ecchymoses occurred distal to the wound, but not elsewhere in the ear. Even 20 minutes after injecting the ear but very little dye escape had occurred from the lymphatics in the region proximal to the incision. In a few instances the channels distal to or beyond the cut and several millimeters from its edge were not only highly permeable while those proximal were not, but the former were often dilated and the latter constricted. In those animals in which dye solution from the lymphatics failed to stream directly into the cut, the slightest increase in the pressure of injection served to make it do so. The resistance to the flow of dye into the wound scemed to be located not at the edge of the wound itself, but 2 or 3 mm. away from it; for once dye had been forced through this region it readily entered the incision.

Fig. 2 shows the result of an injection of dye into the margin of an ear which had been incised 24 hours previously. The photograph was taken 5 minutes after the injection. Although some dye entered the incision, by far the greater part was shunted around it as described. The greatly increased permeability of the lymphatics distal to the cut is shown by the abundant escape of dye from these areas. As yet no dye escape had taken place from the channels proximal to the wound.

In four of the sixteen instances examined in this way, dye solution, injected at the ear margin, not only streamed into the wound but passed on directly through the incised region into the channels beyond. A photograph of the phenomenon is reproduced in Fig. 3. It shows the passage of dye in three channels either directly through the incised area or perhaps immediately under it and very close to it, with ecchymosis of colored substance into the incision. The extreme permeability of the channel walls distal to the incision and the lack of proximal dye escape are plainly to be seen.

Can it be that actual reconstitution of these channels had taken place or were they merely injured and not severed by the cut? The question is not to be answered from a single preparation such as the one shown.

To gain light upon it, the ears of six mice were incised transversely and the ear margin injected with dye. In each instance dye entered the incision through three or four channels but was not directly carried beyond, showing that the lymph channels had been severed. Diagrams were drawn to show the distribution of these channels, with the blood vessels as landmarks. The following day, when the ears were re-examined, the colored fluid in the distal portion of the charted lymphatics had completely disappeared. Dye injections were again made at the edge of the ear of three of the animals, inserting the needle through the same puncture wound and expelling dye in the same spot as before. In all, the same channels previously rendered visible by dye solution were again filled, and in

addition, in each ear two or more channels unseen the day before. In two of the three instances one or two of the lymphatics which had filled on first injection and carried dye solution into the incision, now carried dye through this region into a continuation of the channel on the proximal side, allowing it to flow to the base of the ear. In the cut region profuse ecchymoses of dye solution took place immediately, and distal to the wound the lymphatics allowed dye to escape 3 or 4 minutes later, the normal interval between injection and dye escape being about 10 to 15 minutes. Proximally, no dye appeared outside of the lymphatics in 20 to 25 minutes.

Two of the remaining three animals were examined the next day and the last of them on the 3rd day after incision. In these similar results obtained save that with each day more channels were demonstrable traversing the wound area, and delivering dye solution to their continuations beyond. The evidence showed that the severed lymphatic capillaries reunited rapidly.

In four other mice bearing incisions 24 hours old intravenous injections of pontamine sky blue demonstrated an excessive permeability of the dilated tortuous capillaries and venules about the wound. Within 4 or 5 minutes the region was stained a deep blue by profuse dye escape from vessels there, but not elsewhere in the ear. No patent blood vessels entered the wound as yet. Nevertheless there appeared to be a rapid turnover of fluid within the incision; for, in the instances just described, when dye was brought into the incised region from the ear tip by way of the lymphatics, the resulting colored ecchymoses in the neighborhood of the injury disappeared far more rapidly than elsewhere in the ear.

In ten instances the usual routine dye test was postponed for 48 hours after making the wound. The cut ears were still edematous and hyperemic but the edema fluid was transparent.

In all these animals, after local intradermal injections of standard pontamine solution at the ear margin, dye failed to pass into the incised region. In six of the ten instances, no dye reached the margin of the wound, the lymphatic channels filling to a point 2 or 3 mm. distal to the cut and piping the dye through small lateral channels to other larger lymphatics which passed around it. When the pressure of the injection was increased slightly three or four lymphatic capillaries carried dye freely into the cut.

As in our earlier experiment, the resistance to flow of the dye toward the wound seemed located several millimeters distal to it and might be attributed either to pressure from the edema or to the existence of fibrinous plugs which were dislodged by the pressure. In four of the ten instances in which the pressure of injection was not increased some dye solution reached the incisions directly, colored fluid either pouring into the cut from the open ends of the channels or passing by way of a channel that was now intact, but, with profuse ecchymosis of dye into the cut. It was plain that in the wound area itself the severed lym-

phatic channels had either remained open or had reconstituted. They had not been blocked.

In all of the animals examined 2 days after making the skin incisions, the channels distal to the wound appeared much wider than those proximal, and the bulbous swellings were broader and larger. After entering the lymphatics dye began to escape within 4 or 5 minutes while from the proximal channels none appeared in half an hour.

## The Minute Lymphatics in Later Stages of the Healing of Incisions

Daily, for 10 days, groups of four or five mice, taken from a number with ears incised at the same time, were anesthetized with luminal and examined for changes in the behavior of the lymphatics during the process of wound healing. The early findings differed but little from those already described, such differences as were noted being attributable to variations in the severity of the wounds rather than to the passage of time. A week after incising the skin of the ear edema was still present both distally and proximally to the wounds. The whole ear still showed moderate hyperemia. Under the binocular microscope many new-formed blood vessels were visible extending into the injured area, more of them on the proximal side. On both sides large numbers of tortuous blood vessels with rapid circulation were present, often with reversed venous flow in some of the larger veins.

The introduction of dye into the lymphatics disclosed a variety of changes. The channels distal to the cut were still far more permeable than normally, profuse dye escape through their walls beginning in 2 to 4 minutes after it had entered them. This was observed even 12 and 14 days after superficial cuts had been made in the ears. At this time the skin appeared healed to the unaided eye. The lymphatics appeared widely dilated either as a whole or in segments. The bulbs adjacent to the valves also appeared greatly elongated and widened, giving the appearance of aneurysmal swellings.

In all these experiments the region immediately next the healed edges of the incision was not entered by the dye, save in each instance by three or four channels which transported colored fluid through the incision into their continuations beyond. In passing the region of injury profuse ecchymoses of dye occurred, as in the dye experiments done 2 or 3 days after incising the ears. By far the greater amount was passed into lateral channels about 3 mm. distal to the incision, and by them to lymphatics which ran around the wound. Usually at the extreme ends of the incision where healing had just begun several trunks filled with dye and passed it on toward the base of the ear.

### New Formation of Minute Lymphatics in Areas of Repair

Occasionally in animals tested on the 6th and 7th days, and characteristically in those examined later from the 8th day on, evidence of new formation of lymphatics was obtained.

When a local injection of standard pontamine solution at the margin of the ear was watched under the microscope one could nearly always observe the column of dye-stained lymph advancing down the lymphatics as usual. But a few seconds later, instead of stopping or being carried around the injury, it spread into a close network of very minute channels, lymphatic sprouts, forming a dense reticulum on the distal edge of the incision itself. Scarcely did dye reach these before it began to escape, long before any had come out from the channels in the uninjured portion of the ear. Within 1½ to 2 minutes the semicircle of the distal circumference of the incision assumed a diffuse blue. Such channels were never found in ears examined less than 6 days after an incision.

Fig. 4a shows an ear incised 9 days previously and now injected intradermally at the margin with India ink purified by dialysis. It was amputated at once after injection and photographed while submerged in neutral paraffin oil. At the left end of the incision, one sees several channels which conducted ink through the region of injury. On the right side reconstitution of channels, or a collateral circulation has not yet developed. In the incision itself many very narrow, linear, parallel lymphatics, situated in the new tissue filling the incision are disclosed by the ink. They lie at right angles to the main trunks, having the direction of the original cut. They are better shown in Fig. 4b, an enlargement of the same specimen. Unfortunately India ink, owing to its particulate character, fails to fill as many of the very minute lymphatics as a dye solution and it gives no true idea of the richness of the new-formed plexus. When standard pontamine solution was employed in such experiments it escaped so rapidly from the small vessels that good photographs could not be taken though the abundance of new-formed lymphatics was plainly disclosed.

When local injections of dye were made, 1/2 to 3/4 cm. preximal to the incision, similar networks of newly formed lymphatic capillaries were found in its proximal lip. Retrograde passage of dye occurred showing that the new capillaries were

without valves. They were exceedingly permeable as shown by the almost immediate escape of dye. The old channels on the proximal side of the wound failed to allow dye to escape in half an hour.

To study the relation of the new-formed lymphatics to regenerating blood vessels, the ears of 20 mice were incised in the usual way and after 10 days the animals were anesthetized and injected intravenously with 0.05 cc. of isotonic aqueous pontamine sky blue solution. Two minutes after the injection was completed, a local injection of 2 per cent vital red in the usual menstruum of 1 part mouse serum and 3 parts Tyrode's solution was injected intradermally at the ear margin. The dense plexus of new-formed lymphatics about the wound filled with red dye and the newly formed blood vessels with blue. two types of vessels entered the healing area about equally far and both were much more permeable than those elsewhere in the ear. In the center of the healing wounds neither new-formed blood vessels nor lymphatics were visible. In about half the instances the incisions were traversed by two or more larger lymphatics, which passed directly through or just beneath them. No blood vessels appeared in this area.

## The Lymphatic Capillaries in and about Burned Regions

To study further the lymphatic capillaries in the periods of formation and recovery from inflammation, advantage was taken of the fact that sharply localized, standard burns of similar intensity could be made in the skin of the ear of the mouse.

Injury by Heat.—Anesthetics were used, as already described. To obtain comparable burns we adopted modifications of methods to apply mild heat stimuli to the ear of the mouse (2). Several thin-walled glass water chambers were blown in various shapes and sizes with a flat surface that could be placed against the animal's ear. Each possessed three openings, one for the insertion of a thermometer and two for circulation of water at the desired temperature. One of these chambers possessed a flat oval surface only 3 mm. in diameter. To produce localized burns, hot water was circulated through the chambers and the ears of the anesthetized mice were gently held against the flat surface. By varying the temperature of the water or the duration of contact of the chamber with the ear, first, second, or third degree burns could be produced at will.

"Stigmatic Burns."—Strictly localized, marked superficial burns, which we may term stigmatic burns, were obtained in another manner. An ordinary bac-

teriological platinum inoculating wire was heated at its mid portion by a microburner until the free tip glowed dull red. The etherized mouse lay with the ear resting on the tip of the index finger and the hot wire was quickly and lightly touched to the central part of the upper surface of the ear. Later at intervals the animals were reanesthetized with sodium luminal in groups of ten, as the experiments required.

Stigmatic burns usually healed rapidly in a few days but occasionally progressed to necrosis, resulting in a neat perforation of the ear, as though it had been punched out.

The burns produced by the small water chambers and the hot platinum wire involved but a small part of the animal's ear, at its middle.

Changes in the permeability of the blood vessels were determined as described earlier in the paper. To test lymph permeability, standard pontamine solution was employed in the usual manner.

The local injections of standard pontamine solution into the tissue at the margin of the ear resulted in its entrance into those lymphatic capillaries which lay in uninjured tissue but which passed through the burned regions and emerged again into normal tissue. Other channels not directly involved in the burn but surrounding it, both near and remote, also took up the dye solution. Control observations were made by injecting the uninjured ears of the same animals. As the processes of repair were often protracted, dye injections were made soon after injury in some instances, in others at half hourly intervals up to 6 hours,—and, finally, at daily intervals up to 10 days or 2 weeks. Always before injecting the standard pontamine solution, the condition of the circulation of the ear was determined under a binocular microscope. The mice lay in plastalene moulds with the ears lying horizontally on porcelain placques and illuminated as previously described (3, 9).

Experiments were done on four series of mice with the water chamber at 46.0°, 53.0-55.0°, 59.0-60.0°, and 67.0°C., respectively. In yet another large series of animals stigmatic burns were produced. It was found that increasing degrees of heat or a longer application simply increased the severity of the reaction. When the burns were severe enough to cause permanent arrest of blood flow the lesions progressed to necrosis within 36 to 48 hours. Such burns almost invariably followed application of the chamber to the ear for 1 minute with water circulating at 59.0-60.0°C., or for as short a period as 20 seconds with the water temperature at 67°C.

When a small spot of skin on the mouse's ear approximately 3 to 5 mm, in diameter was heated at 55°C, for approximately 1 minute moderately severe burns resulted. In the sharply localized region of injury circulation in the smaller vessels was stopped, and often, even in the largest radial veins and arteries, blood flow ceased temporarily. A few seconds afterwards a reactive hyperemia occurred about the heated area, which in 2 or 3 minutes extended over the whole ear. After a few minutes, pronounced edema formed in and about the burn, that is to

say the ear became thicker there, the skin tense, and, to a blunt needle, there was "pitting on pressure." Puncture with a sharp needle led to the escape of fluid, and under the binocular microscope the edematous area assumed a ground glass appearance.

The Immediate Effects of Severe Burns.—When standard pontamine solution was injected into the outer margin of the ear, a few minutes after heating an area midway between the tip and the base, an extraordinary increase in the permeability of the walls of the lymphatics in the burned area was manifest. Dye passing into them from the normal channels nearer the margin at once escaped through their walls into the surrounding tissue.

Fig. 5a shows the escape of dye from the lymphatics in a burned area only 2 minutes after dye injection into an ear submitted to the water chamber at 55° for 40 seconds, 8 minutes previously. Such contact usually resulted in a third degree burn with temporary stoppage of the circulation for several hours and severe edema.

The region of dye escape from the channels coincides with the burned area. From the lymphatics in the uninjured portion of the ear there has been no escape of dye. In Fig. 5b the same preparation is shown 2 minutes later. Much more colored matter has now escaped from the channels in the burned area and it is distributed more widely.

In ten instances dialyzed India ink was injected together with standard pontamine solution into the margin of ears which had been burned in the same way. Although dye escape in the injured region was prompt and abundant the ink particles failed to pass out and the lymphatic capillaries were clearly defined by it.

Fig. 6 illustrates the immediate findings in a burn of lesser degree when there had been time for the formation of edema. The water chamber at 55°C. was in contact with the ear for 20 seconds. An hour later the standard pontamine solution was injected, the photograph being taken after another 4 minutes. The area of the burn, which corresponds with that of the escape of dye from the lymphatics, was already edematous, yet the channels penetrating it carried much of their contents into the uninjured tissue beyond, where no dye escape from the lymphatics occurred. Ink escaped from none of the lymphatic channels of such burned areas.

When the chamber containing water at 67°C. was applied to the ear of the mouse for 30 seconds or more, or when the ear was touched

PHILIP D. MCMASTER AND STEPHEN S. HUDACK with the hot platinum wire, marked punctate burns resulted. the experiments in which moderate burns were induced, so in these, stasis in the injured area and reactive hyperemia round about occurred

When isotonic pontamine sky blue was injected intravenously 10 to 20 minutes after a pronounced contact or stigmatic burn, the blood immediately. Edema followed. vessels surrounding the ischemic burnt area were unusually permeable. Within 2 to 4 minutes after the injection, there could be seen about this area a collar of deeply stained tissue, colored by the escape of dye from the blood capillaries and venules close to the burn and long before escape from blood vessels elsewhere in the ear was visible. The tissue within the burnt region itself remained ischemic and therefore

In contrast with these findings, following a local intradermal injection of dye at the margin of an ear burned in the same way, the lymphatics passed much of the colored fluid into the injured region no dye entered it. itself where it escaped from the channels.

In twelve instances, at varying intervals from 3 minutes to 13 or 2 hours after producing the burn, standard pontamine solution was injected into the skin of the ear, close to its outer margin. In more than half of these, lymph channels could be made out traversing the burned area, but the escape of dye from the portions of these within the burns was so immediate and so great that one might almost suppose that the cellular membrane of the lymphatic wall no longer existed as such. Suppose that the centuar memorane of the symphatic wan no longer existed as such. Yet, when the car was reinjected, in five of these instances with dialyzed India ink or "Hydrokollag," the walls of the lymphatics were demonstrated to be still continuous, for none escaped. In three other experiments standard pontamine solution and India ink combined, injected 2 hours after the production of the burns, was seen to enter the lymphatics as usual; and these carried both dye and India ink through the punctuate, burned area into the lymphatics beyond, though there

When the time interval between the production of the burn and the dye injection was increased to 4 or 6 hours, due often failed to reach the ischemic injured was some dye loss during the passage. area by way of the lymphatics. The dye was carried to within 2 or 3 mm, of the hurn and there immediate and profuse ecchymosis of it occurred. The channels which passed around but close to the burn were extremely permeable so that it was nature passed around our case to the pain were extremely be included burn itself surrounded by a dense cloud of diffuse blue color, while the edematous burn itself remained colorless. In two instances a lymphatic channel entrying dye did peneremained coloniess. In two manances a symplantic colonies energing opening to trate the burn and pass through. As result the patch was stained blue owing to the escape of dye from the lymphatic within it.

When dye escapes from lymphatic capillaries which lie outside an edematous burned area the diffuse blue color it gives to the tissue does not extend into the burn. When, however, dye is carried by a lymphatic into the edematous area, it escapes readily from the containing vessel. The edema fluid would seem to be partly made up of lymph which has escaped from lymphatic capillaries traversing the injured region.

Figs. 7a and 7b are photographs of an ear taken 3 and 8 minutes, respectively, after a marginal injection of dye. Three hours prior to this a third degree punctate burn was made (the water chamber for 45 seconds with water at 60°C.). In the first photograph, taken only 3 minutes after injecting the ear, the pale region of the burn had become edged with blue dye owing to escape from the lymphatics at its edge. A single large one carries dye directly through the burn itself and the dye escaping from it has merged with that from the ecchymoses on the right. The second photograph taken 5 minutes later illustrates the progress and extent of dye escape. After burns such as this the entire ear structure remains hyperemic for several days. Capillary dilatation in the region surrounding the burn is pronounced and the edema progresses until the ear may be half a centimeter thick. Under the microscope the blood vessels appear as if seen through ground glass.

Heating the skin to 67°C. for 15 seconds frequently gave rise to blisters. If dye was injected into the ear margin just beforehand, a very pronounced escape of dye took place from the lymph channels. The extravascular movement of dye under these circumstances was almost immediate, and it frequently extended several millimeters from the channels. The observation suggests that ordinary blister fluid is formed not only from vascular transudate but from a lymphatic one as well.

The findings as a whole furnish evidence for the participation of the lymphatics in the formation of and recovery from edema, confirming the observations of an earlier paper (2). Further work on this theme is now in progress.

# The Lymphatics in the Early Stages of Restoration and Repair of Superficial Burns

Under ether anesthesia, stigmatic or contact third degree burns were made on one ear of a large number of etherized mice. Each day thereafter groups of eight or ten animals were anesthetized with sodium luminal and a dye injection made intravenously or into the ear margin. As controls the normal ears of the same animals were used.

The findings during recovery from both types of burns were so similar that no attempt will be made to distinguish between them.

Twenty-four hours after the formation of stigmatic burns, each was surrounded by an area of pronounced hyperemia and edema. Under the binocular microscope the whole ear showed a mild reactive hyperemia with dilatation of capillaries and venules about the burn and severe edema at the margin of the latter. Stasis in the blood vessels round about varied in degree, but in all instances the region of actual injury was ischemic.

In these instances and in experiments to be detailed there were many findings similar to those occurring after incision of the ear. After a burn, as after incision, there was a strong tendency for dye injected at the ear margin to be carried around the region of injury by numerous enlarged lymphatic channels. During the first 24 hours after the injury these, when they contained dye, showed a greatly increased permeability, with profuse ecchymotic dye escape wherever a lymphatic approached the injured region itself.

Occasionally, lymphatics transported colored fluid directly into and through the burned areas as through incisions. Though the areas were edematous, dye escaped so rapidly into them that one might doubt the existence of lymphatic walls, were it not that India ink did not escape. However, in most cases the channels surrounding the burn led most of the dye around it. Those immediately next the burns seemed to terminate in blind ends which may have been closed, perhaps by heat coagulation or thrombosis. Only when the injections were made with some pressure was colored fluid forced into the burn itself. Then, if such a burn had been made 2 to 6 days previously, the lymphatics failed to carry dye completely through it into the proximal normal tissue, but instead an ecchymosis of dye resulted within the injured area itself.

A further similarity in the reaction of lymphatics after injury by burns and incision was observed in the fact that 24 to 30 hours after burning the ear the lymphatics proximal to the burn appeared as sharply outlined bands showing no escape of dye into the surrounding edematous tissue, even 20 to 25 minutes after an injection. The color within the channels became rapidly paler as though the contents had been diluted and cleared by an intake of fluid. Instead of the excessive escape of dye from the lymphatics within the burn and distal to it, there was an apparent failure of dye escape in the proximal tissue.

## The Lymphatics in the Later Stages of the Healing of Burns

Two days after a burn had been made, the lymphatics which filled most readily, that is to say those which skirted the burn, lay within the hyperemic area surrounding the injury. Little or no apparent escape of dye occurred from them, although they were usually somewhat dilated. The contents were more rapidly carried away than in normal channels, to judge by the fading of the blue color within them, and without visible dye escape. The few ecchymoses which did occur in the region disappeared in 10 to 15 minutes, but distal to the injury they often persisted for several hours. The observation afforded further evidence of an increased lymphatic turnover going hand in hand with hyperemia and increased vascular permeability.

In five instances this last was easily demonstrated by intravenous injections of dye solution. Dye poured from the dilated capillaries and venules about the burn long before it escaped in quantity elsewhere in the ear. As result a bright blue ring about 4 mm. thick was formed surrounding the colorless area of injury. Fig. 8 shows the result of such an experiment, photographed 4 minutes after the intravenous injection was made.

On the 4th day, local and intravenous injections of dye into such animals yielded findings similar to those just reported.

The blood vessels seemed still to be more permeable than normal, judging by the rapidity of dye escape, but one could not say whether this was due to a more permeable state of the vessels or to an increased supply of stained blood. The lymphatics too were more permeable than normal in the region distal to the burn, and here they seemed wider than those proximal to it. When dyes entered the channels completely surrounding the burned area, profuse escape occurred distally, forming a blue cresent of diffuse staining. The proximal lymph vessels seemed less permeable than normal, for within half an hour after their injection the bluestained contents had become pale, without showing any visible escape of dye. Whether the failure of dye escape is due to decreased permeability of their walls or to brisk fluid movement into the vessels from without cannot be said. In either case the phenomenon shows drainage of the tissues by the lymphatic capillaries.

The findings in no way differed 5 days after producing the burns but by the 6th and 7th day the inflammation seemed to have subsided. No longer was there evidence of hyperemia in the ears, save for a few dilated capillaries immediately about the injured area. The edema had resorbed, leaving only a small swollen region in and about the burn, which by this time had become indurated. Nevertheless the usual local injections of pontamine solution showed the lymphatics to be still excessively permeable. Between the burned area and the ear margin numerous local ecchymoses of dye occurred, as profusely and rapidly as on the 1st and 2nd day after injury.

### New Formation of Lymphatics within Burns

By the 7th or 8th day of healing evidence was obtained of new formation of lymphatic capillaries, as in our experiments with incisions.

When dye injected at the ear margins reached the burned areas, some of it entered a dense, ramifying, twig-like plexus of lymphatic capillaries within the distal edge of the burn itself. Some dye passed through in large channels, the remainder was carried around the injury. Fig. 9 shows such a new-formed network about a burn 9 days old. The photograph was taken 2 minutes after injection of the ear with a suspension of India ink. Only a few of the fine, hair-like projections extending into the new-formed tissue are visible; for ink suspensions did not yield a complete injection of these vessels.

To show the relationship of the new lymphatic capillaries to blood vessels intravenous injections of pontamine blue solution were followed in 1 or 2 minutes by injections of vital red in the ear tissue, as in our earlier work with incisions. The findings were so much alike no separate description will be given.

Similar experiments were made during the later stages of healing. Lymph channels were found completely traversing the burns. The sequence of events when dye was introduced into them has already been described.

#### DISCUSSION

In our previous papers attention has been called to alterations in the permeability of the lymphatic walls occurring under the conditions of the everyday life of the animal. Even very mild thermal and chemical stimuli result in the escape of substances of large molecule, for example hemoglobin, which are retained within the lymphatics of an unharmed ear. On the other hand true particulate matter, for example India ink, fails to escape, showing that no lacunae exist in the lymphatic wall. In the present paper we have reported upon the changed conditions in the lymphatics following incision and frank injury of other sorts. Profound alterations in the permeability of lymphatic walls, in and about these injured regions, speak for an active participation of the lymph system in the changed processes of fluid exchange. What can be inferred concerning the rôle of the lymphatics during inflammation and repair?

Our experiments make plain the fact that, like the blood vessels, the lymphatics respond to injury first by pouring their contents into the region involved. During the first few minutes or even hours after an injury the permeability of the lymphatic wall is enormously increased without loss of its anatomical continuity, at least under the conditions of the experiments described. The most indiffusible vital dyes pass out readily but ink particles are retained. The lymphatics are so permeable that it is difficult to suppose that they can still function as channels for the conveyance of fluid. That they fail to do so adequately is indicated by the developing edema. phatics are rendered more permeable for a considerable distance around the immediate region of injury. Inflammatory edema is obviously due to a change in the lymph vessels as well as in the blood vessels. The nature of the various changes is not understood. Soon after a burn the lymphatic capillaries distal to the wound appear dilated and those proximal either constricted or normal,—the former more permeable than the latter. The increased permeability may follow dilatation. Yet obvious changes in permeability have been observed which appear to be functional and not anatomical. Thus, the lymphatic capillaries, skirting a burn or wound and not directly affected by it, neither dilated nor constricted, are usually for the first 48 hours much more permeable than normal.

Our work indicates that the brief isolation of a skin region following injury, an isolation resulting from the excessive permeability of both lymphatics and blood vessels, is first lessened when the lymphatics begin to function again. Their severed ends, unlike those of the blood vessels, remain open and lead away fluids from the wound. The fact that lymphatics remain open and the blood vessels closed in an incision goes far to explain the frequent infection by way of the lymphatics. Soon after an injury in the skin of the ear, the lymphatics lying between the head and the injured region regain their normal permeability and apparently function as draining and conducting channels. Through these and not through the blood vessels resorption from the wound first begins. Through the lymphatics toxins or noxious products of disintegration are first carried away to be sieved or passed through the lymph glands before reaching the body at large. The evidence for this fact, though by no means complete, is sufficient.

In this connection, it should be recalled that we have been dealing with a type of lymph drainage slightly different from that in human skin. The ear of the mouse is very thin and both the superficial and

deep plexuses of lymphatics lie close to the surface. As result the lymphatics usually injected lie in the deeper plexus. It is possible that the current in these channels is more rapid than in the superficial lymphatics of the skin of man, that is to say, the lymphatic capillaries which become filled by intracutaneous injections of dye (1). There results in the mouse ear a lymph current just beneath the epidermis which probably does not exist in the human skin, for there the intradermally injected dye passes, after running 2 or 3 cm. in the superficial channels, into a deeper layer (1). However this may be, the fact should be stressed that the lymphatics injected in these experiments with mice probably correspond in function with the deeper plexus found in the subcutaneous tissues of man, lymphatics which are sufficiently superficial to be involved by any cut causing hemorrhage, or by a third degree burn. In these deeper lymphatics in man there is, of course, a considerable flow (1).

The walls of the lymphatics directly draining a burned or incised area, after having at first been abnormally permeable, within 48 hours after the injury became far less permeable than normally for diffusible substances. At this time intravenous injection of dye shows the blood vessels in the injured region to be still abnormally permeable and, owing to hydrostatic conditions, they must still be adding to the accumulation of tissue fluid. The tissue is edematous and rapid continuous passage of fluid must be taking place through the lymphatics. for dve solution placed within them quickly pales and is swept away. In the later stages of healing, 48 to 72 hours after an ear injury, there is a definite tendency for lymph peripheral to the injured spot to flow around it, instead of through it. Dye injected into the distal channels is carried to a point close to the injury and then is shunted around it. It is as though the lymphatics were obstructed by wound materials or perhaps plugged, as has been suggested by Menkin (13) in his work upon blood vessel changes in large areas of inflammation. Evidence of a participation of the lymphatics in repair is seen in their tendency to rapid reconstitution and a new formation of them not only in the regions where they have merely been severed but where they have been injured or destroyed by burns.

Great numbers of new lymphatics were seen in the regions recovering from burns. When the latter had been severe enough to cause necrosis

of the ear and perforation many new tiny lymph capillaries were found in the granulation tissue of the healing margins obviously taking their place in actively growing tissue. But in the tissue far removed from the healing margins, though still within the burned or incised regions, as shown in Figs. 4a and b and Fig. 9, many more lymphatic channels became visible after dye injection than in similar areas of normal ear. As the photographs show, some were large and some small. earlier paper (3) we have shown that the superficial lymphatic plexus is only partially filled by dye injections, that just as Krogh has shown for blood capillaries, small lymphatics temporarily closed and invisible may be teased open to allow dye to enter. As result one cannot say whether the excessive number of dye-filled lymphatic capillaries in the burned area signified a new formation of channels or a flooding of the entire lymphatic plexus, usually but partially visible after intradermal injection of dye and now entirely filled in a tissue engaged in active fluid exchange. When such burns were examined several days later, the number of dye-containing lymphatics had decreased to the normal number. Whether the channels were cut off or had closed down in a relative period of rest, cannot be said.

The observation that new collateral lymphatics can form is of course not new. It occurs after ligature of the thoracic duct (14, 15) and of large lymphatics of the limbs (16). Regeneration of lymphatics in the ear of the rabbit has also been demonstrated (17) as further their growth into granulation tissue (18). Union of lymphatics has been described by E. R. Clark (19) in the tadpole's tail and by Clark and Clark in transparent chambers in the rabbit's ear (20, 12).

In the past the speed with which a new formation of lymphatics occurs has not been recognized nor has it been appreciated that lymphatic drainage may be instituted from areas of repair before drainage takes place by way of the blood vessels.

#### SUMMARY

With the aid of solutions of vital dyes the lymphatic capillaries in the ear of the mouse have been studied during the period of immediate reaction to injuries of various sorts and during the period of repair.

The behavior of lymphatics severed by incision differs greatly from that of the small blood vessels. Instead of closing they sometimes remain open for as long as 48 hours. Materials introduced into the wound pass directly into the lymphatics through their gaping ends, a fact which will explain why infection from incisions is predominantly

All around an injury the lymphatics are rendered abnormally permeable. So, too, are the blood vessels, a fact well recognized in the along the lymphatics. past. Twenty-four to 48 hours later, at a time when the blood vessels in the edematous tissue surrounding the injured area are still much more permeable than normal, the draining lymphatics allow far less to escape than usual. The possible reasons for this have been discussed. The lymphatics participate in the removal of fluid from the edematous tissue.

As repair after injury takes place severed lymphatics may reunite when as yet there are no functioning blood vessels. Later an active hyperplasia of the lymphatic channels occurs, an extraordinarily abundant plexus of minute lymph capillaries budding into the area under repair. BIBLIOGRAPHY

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#### EXPLANATION OF PLATES

#### PLATE 28

- Fig. 1. Photograph of the ear of a living anesthetized mouse, injected at the ear margin with standard pontamine solution, 5 hours after a transverse incision had been made in the skin of the upper surface. The dye entered the lymphatics of the injected area and gradually extended along them to escape from their severed ends, filling the wound with blue dye. ×6.
- Fig. 2. The result of an intradermal injection of standard pontamine solution into the margin of a mouse's ear, which had been incised 24 hours prior to the injection and photographed 5 minutes after it. The blood vessels and lymphatics had been cut through. Some of the dye reached and entered the incision but most of it was shunted around it as described in the text. The lymphatics are markedly permeable distal to the incision, as indicated by the abundant dye escape there, and much less so proximally. ×10.
- Fig. 3. Lymphatics in the incised ear of a living mouse photographed 5 minutes after an injection of standard pontamine solution. The incision was made the day before and is easily seen in the photograph. Three lymphatic channels have conducted colored fluid past the incision into the tissue at the base of the ear. In doing so much dye has escaped, either into it or just beneath it. The increased dye escape distal to the incision, and the lack of escape proximal thereto is also well shown. ×10.
- Fig. 4a. Demonstration with India ink of the lymphatic plexus about a healing wound. Nine days previously the skin had been incised; healing was progressing well. Immediately after a marginal injection of ink the ear was amputated and photographed under neutral paraffin oil. On the left side of the incision several lymphatics are seen carrying ink either directly through the healing incision or just beneath it. In the injured area a few very fine lymphatics can be made out. Linear and parallel, they lie transversely in the healing incision itself. ×10.
- Fig. 4b. An enlargement of the same specimen. In this figure these newly formed lymphatics are seen to better advantage.  $\times 40$ .

### PLATE 29

- Fig. 5a. This photograph shows the escape of pontamine solution into a burned region. Eight minutes prior to taking the photograph a chamber containing water at a temperature of 55°C. was brought into contact, for 40 seconds, with an area midway between the tip and base of the ear. Six minutes later the dye injection was made near the tip and 2 minutes later the photograph was taken. The area of dark, fuzzy escape of dye along the lymph channels coincides with the area of the burn. Elsewhere no dye has passed out. ×9.
- Fig. 5b. The same preparation 2 minutes later, that is to say 4 minutes after the injection. In the unharmed car standard pontamine solution does not begin to escape from the lymph channels for 10 to 15 minutes. ×9.

Fig. 6. Ear burned in a spot midway between the tip and the base by 20 seconds contact with the water chamber containing water circulating at 55.0°C. One hour later standard pontamine solution was injected at the tip of the ear and 4 minutes afterwards the photograph was taken. The lymphatics, though allowing some escape of dye into the burned region, where it colors the edema fluid, have transported much of it into the proximal, normal tissue. ×20.

#### PLATE 30

Figs. 7a and 7b. Two photographs of the same preparation taken 3 and 8 minutes respectively after a local injection of pontamine solution at the margin of the ear. Three hours prior thereto a severe third degree punctate burn had been made midway between the tip and the base of the ear as described in the text.

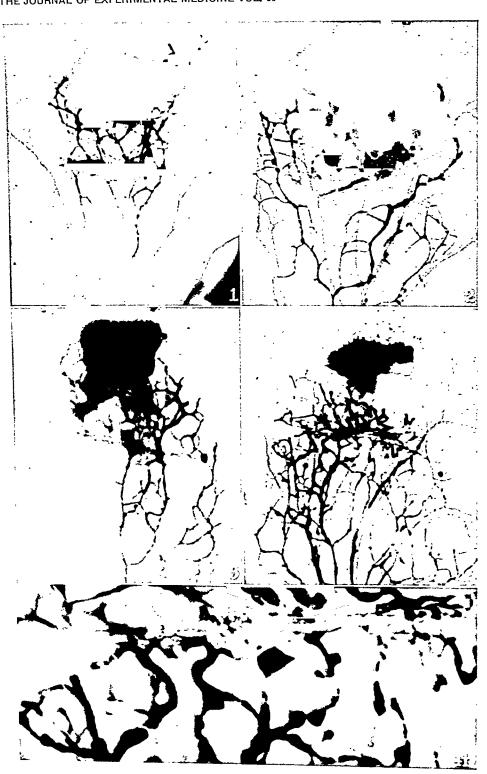
The rapid dye escape from the lymphatics close to the burned region is shown and the fact that the latter is almost unstained. A large channel traversed it from which dye ecchymosis occurred after a time. ×6.

Fig. 8. A photograph of the ear of a living anesthetized mouse 2 days after a stigmatic burn had been induced on its upper surface as described in the text. Four minutes prior to the photographic exposure the animal received intravenously 0.05 cc. of 21.6 per cent aqueous isotonic pontamine sky blue solution.

The increased permeability of the smaller blood vessels is evidenced by a ring of intense color about the burn, while elsewhere in the ear very little dye has escaped. At the center of the burned area there is some slight diffuse staining. ×6.

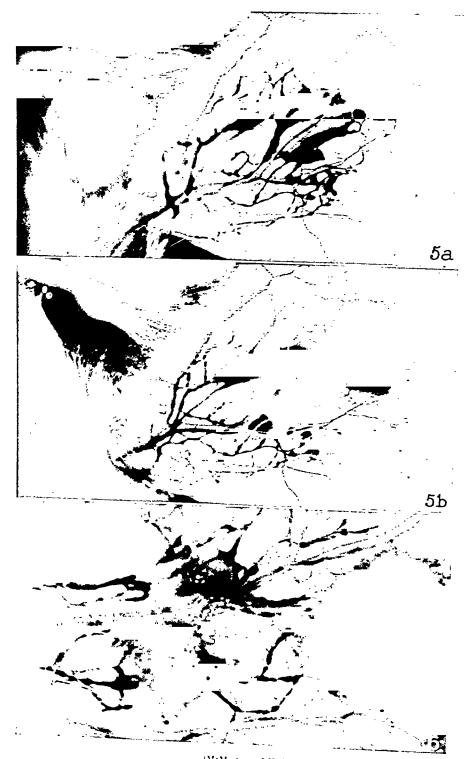
Fig. 9. The ear of an anesthetized mouse injected with a suspension of dialyzed India ink in 5 per cent gelatin solution 9 days after a stigmatic burn. One minute after the injection the ear was severed, placed under neutral paraffin oil, and photographed after another minute. The burn had caused a complete perforation of the ear which at the time of the injection was gradually being closed by granulation tissue. Several very small new-formed lymphatics can be seen in the new-formed tissue and about the healing burn there is an abnormally rich plexus of lymphatics many of which are very small. ×6.





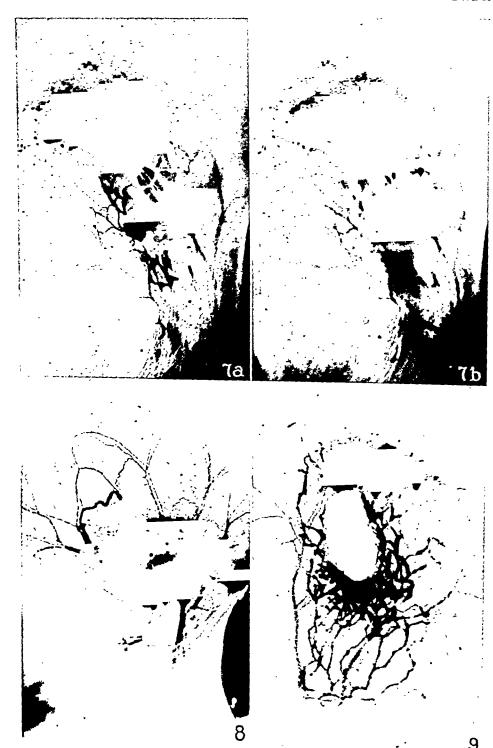
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# IRREVERSIBLE CHARACTER OF THE LATE CHANGES AFTER HEPATECTOMY

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(Received for publication, May 31, 1934)

Death invariably follows extirpation of the liver in mammals. The first symptoms which arise are due to hypoglycemia, and vanish upon administration of glucose (1-5). For several hours, if sugar is given, the animals show no symptoms but later, despite all treatment, a second clinical stage develops, characterized by ataxia, weakness, coma and respiratory failure and ending in death (4, 5). The sequence of events is the same in the rabbit as in the dog (6-8). As the cause of the fatal issue is unknown, the work to be reported here was undertaken in an attempt to gain insight into it. We have endeavored to learn whether rabbits manifesting the symptoms of the advanced or "second stage" of liver insufficiency can be aided by the circulation of their blood through the livers of healthy animals or by crosstransfusion with normal animals. A preliminary note based upon some of this work has already been reported elsewhere (9).

## Results of Circulating the Blood of Liverless Rabbits through a Normal Liver

Control Experiments.—Before attempting experiments with hepatectomized animals, trials were made with etherized normal ones. A carotid artery and external jugular vein were connected with the portal vein and hepatic vein respectively of the freshly removed livers of other rabbits and the blood was allowed to run through the organ for periods ranging from 2 to 6 hours. The technique will be described below. During the tests flow was free through the livers and the current in the intralobular capillaries could be observed with a microscope. The large amounts of bile secreted by the transfused organs gave evidence of their functional integrity. Sections of the hepatic tissue taken at the end of the experiments showed no changes from the normal appearance; and the healthy rabbits which furnished blood showed no ill effects whatever.

We next endeavored to determine whether the passage of liverless animals' blood through the liver of a normal rabbit would bring about



observer was in constant attendance upon the animals, the temperature was regulated by turning the light on or off.

From time to time the pupillary and musculo-motor reflexes were examined. At these times, before the second stage symptoms of liver deprivation had begun they were normal, and the pulse, respiration and temperature, recorded at intervals of a few minutes through all the experiments, were normal too.

While the liverless rabbit was still in the first stage, blood specimens from four male rabbits of about the same size were taken and examined for cross-agglutination with the blood of the liverless animal. From amongst the four, three donors with compatible blood were readily selected. Incompatibility is rare in rabbits.

The approach of the second stage after hepatectomy was usually first detected by a change in the animals' posture. During the first stage hepatectomized rabbits receiving the necessary injections of dextrose sit as do normal animals, with elevated head and the paws drawn well under the body. At the approach of the second stage one notices a drooping head and the paws tending to sprawl apart. The animals seemed startled when touched and soon showed ataxia if placed on the floor and allowed to move about.

With the development of these early signs in the liverless animal with blood sugar artificially maintained at a high level, two of the selected donors were bled 30-35 cc. The blood was defibrinated and placed in a water bath at 39°C. When a short time later the signs of the second stage of liver insufficiency had become clearly manifest in the liverless animal a third, freshly fed donor rabbit was anesthetized with ether and injected intravenously with 5 cc. of 1 per cent heparin solution. Paraffined cannulae, filled with the defibrinated blood and plugged, were swiftly placed in the portal vein above the juncture with the splenic vein and also in the vena cava just above the entrance of the renal vein. A third cannula was inserted into the bile duct after tying and cutting the cystic duct. The thorax was then opened and a ligature rapidly thrown about the inferior vena cava above the diaphragm. The liver, with cannulae in place in the extrahepatic portions of its vessels, was removed together with the portion of diaphragm surrounding the vena cava. The whole was immediately submerged in a bath of warm paraffin oil at 39°C. contained in a soft basin of rubber dam tissue fixed within the rim of an iron ring-stand. At once thereafter the cannulated carotid artery of the liverless rabbit was connected with the portal vein cannula of the liver preparation, and the jugular was hitched to the inferior vena cava. This was done by means of short paraffined rubber tubes which, together with the cannulae, were filled with defibrinated blood and clamped. The tube leading from the carotid artery bore a side arm connecting with a mercury manometer for blood pressure readings. 3 cc. of 1 per cent heparin solution was added to 15 cc. of blood from one of the donor rabbits and the tube filled with this and clamped between the manometer and the liver preparation. Now, 25 cc. of the blood of the first or second donor rabbit was slowly injected through the rubber tubing beyond the clamp in the direction of the portal vein of the liver preparation, together with 5 cc. of 1 per cent heparin solution. This was done to charge the liver preparation with blood, thus preventing subsequent loss of blood from the liverless animal into it. No fall in blood pressure of the hepatectomized animal resulted when heparin was injected first into the liver preparation and distributed to the blood in this way. on the vena cava-jugular vein connection was released and at once a flow of blood occurred from the liver toward the hepatectomized rabbit. The remaining clamp on the carotid artery-portal vein connection was next removed and circulation through the excised liver commenced. As result the arterial blood passed through the portal vein to the liver lobules, and reaching the vena cava by way of the hepatic vein, entered the liverless rabbit once again. The upper end of the vena cava had been ligated and so too had the hepatic artery. Excessive flow of blood from the carotid artery, with resultant distention of the liver with blood, was prevented by partial occlusion of the tube attached to the carotid cannula. The temperature of the oil bath containing the liver was maintained at 38-38.5°C. by constantly stirring in additions of paraffin oil at 40.5°C. and draining an equal quantity from the basin. The liverless rabbit remained on the warm pad under the canopy of cotton wadding. Pulse, temperature and respiratory rate were constantly observed.

Blood flow was usually visible in the cannulae but at intervals the occurrence of flow through the liver was confirmed by pinching the tube leading to the jugular vein. Under these circumstances the livers filled with bright blood in about 20 seconds when full flow from the artery was permitted, and emptied in 40 seconds when it was cut off. Bubbles of lymph were seen escaping from the cut lymphatics of the liver, under the oil, and bile was copiously secreted, as shown by the flow from the cannula in the bile duct. In the four experiments of this nature the livers were transfused from 13 to 4 hours. To terminate the transfusion the connecting tubes were simply cut and plugged, avoiding all traction on the cervical nerves. A blood specimen of the hepatectomized animal was then taken at once for sugar determination and an intravenous injection of warmed isotonic glucose was given without awaiting the blood sugar findings. The pupillary and musculomotor reflexes were examined and the animal was observed for ataxia, weakness, blindness and other symptoms of second stage liver insufficiency. It was kept on the warm pad until death occurred, given ample glucose, and pulse, temperature and respiration were observed at short intervals. Careful autopsies were performed to determine whether hemorrhage, insufficient collateral circulation or any cause of death could be found, other than absence of the liver. Blocks were taken from the different lobes of the donor liver. Invariably the sections from these were completely normal in appearance.

The transfusion of the blood of the liverless animals through normal livers produced no clinical improvement whatever, although the functional integrity of the donor liver was evidenced both by the formation of much normal looking bile during the experiments and by the lack of histological change of the hepatic tissue. In each of the experiments some 6-8 cc. of clear green bile was obtained from the cannula in the bile duct. Furthermore, the flow of blood through the transfused livers was always excellent. As soon as the liver transfusion was begun the donor liver became bright red and swollen; but the blood coming from it was distinctly darker than that entering. Partial occlusion of the recipient's carotid artery produced prompt reduction in the liver's size and a change in color to a darker hue.

## Cross-Transfusions between Normal and Liverless Animals

Since "liver transfusion" failed to ameliorate or reverse the symptoms of advanced liver insufficiency it seemed wise to resort to crosstransfusions between liverless animals and intact normal ones. procedure introduces many influences not present in a "liver transfusion;" and if it were carried out soon enough after hepatectomy there should be no reason for the liverless rabbit to develop any insufficiency. We purposely delayed it until the second stage of liver insufficiency had set in. Under these circumstances an opportunity was provided for amelioration through an elimination of toxic substances by way of the intact liver and the excretory organs of the donor. One was also afforded for the observation of possible effects of the cross-transfusions upon the donors, from which one might infer the accumulation of toxic substances in the blood of the liverless rabbits. In many ways the symptoms of the rabbit dying of liver insufficiency suggest that there exists a toxic depression of the central nervous system (4). To explore this possibility some of the crosstransfusions were so carried out as to prevent the dilution of the recipient's blood before it should reach the central nervous system of the donor. In these, as will be described below, the blood leaving each animal from a cannula in the proximal end of the carotid artery entered the circulation of the other through a cannula situated in the distal end of the carotid. In the ordinary experiments cross-transfusions were performed from the carotid artery of one animal to the jugular vein of the other and vice versa.

Control Experiments.—Four cross-transfusions between pairs of equal sized normal rabbits were performed under ether anesthesia. In two experiments, after testing the bloods of the prospective partners for compatibility, paraffined

cannulae were inserted in the left carotid arteries and left jugular veins of each animal and connected by short paraffined rubber tubes 3 cm. in length in such a way that the carotid blood of one animal flowed into the jugular vein of the other. In the other experiments two cannulae were placed in the left carotid artery of each of the animals and connected by rubber tubes in such a way that blood flow from the proximal end of the carotid artery of each one passed into the distal end of the carotid of the other. To prevent clotting in the cannulae prior to the transfusion they were filled with heparinized blood and stoppered as in the previous work. 5 minutes before each experiment was begun both animals received intravenously 3-5 cc. of 1 per cent heparin solution. The cross-transfusions were allowed to continue 1, 2, 3 and 4 hours respectively. No untoward symptoms developed. Only insignificant changes in pulse and respiratory rate were observed. In one of the experiments the effect of bleeding one animal into the other and vice versa was repeatedly tried, by temporarily clamping the carotid artery of one. When the blood pressure of the bled animal fell to about 10 mm. of mercury that of the other rose but 15-20 mm. above its "normal" under the conditions. Both pressures returned to the previous level when the cross-flow of blood was again permitted.

Cross-transfusions were next carried out between normal rabbits and liverless ones in the second stage of liver insufficiency. The liverless animals at times showed slight improvement, perhaps owing to better circulation of the blood, for the skin often became more pink and warm, but, though the cross-transfusions were continued, the animals lapsed rapidly into their previous state.

## Methods and Technique

Rabbits of 1800-2000 gm. were submitted to a preliminary operation (7) to develop collateral circulation about the liver, and later, under ether anesthesia, the liver was removed and two cannulae inserted in the vessels of the neck. Both were placed in the same carotid artery in some experiments, one directed toward the heart, the other toward the head; and in other experiments one in the carotid artery, toward the heart, the other in the jugular vein. They were filled with 10 per cent sodium citrate solution and closed off with pieces of glass rod as usual.

Save where special mention is made the further postoperative treatment of the liverless animals was as in the previous work. Warmed glucose was given by mouth the first night after operation and on the following day intravenously, frequent blood sugar determinations serving as guide to the amounts to be given. As routine, but more frequently than in the instances used for "liver transfusions," the blood pressure of the animals was taken from the carotid cannula,—at hourly or half hourly intervals,—to determine the pressures at which the later cross-transfusion should be done.

For the transfusion two donor rabbits of about the same size as the liverless recipient were selected by cross-agglutination tests for compatibility. From one 40-50 cc. blood was taken with light ether anesthesia from the carotid artery, aseptically defibrinated and kept in a water bath at 39°C., ready for use as will be described below. The other donor was lightly anesthetized with ether and two cannulae were placed in the neck vessels, the location depending upon the type of cross-transfusion to be done.

After hepatectomy about half the rabbits passed into the second stage of liver insufficiency without significant changes in blood pressure as measured from time to time by a mercury manometer brought into connection with the cannula in the carotid artery.

The others showed a marked fall prior to the onset of the second stage. As the experiments demanded postponement of the transfusions until the animals were manifestly in this stage, and as we did not wish to mistake an ordinary improvement in blood pressure resulting from transfusion for an improvement in general condition due to relief from the hepatectomized condition, the liverless animals with low blood pressure were given injections of the defibrinated blood in sufficient amount to maintain the blood pressure until the symptoms of the second stage had fully developed. At times 25-40 cc. was required, given slowly at intervals, in 3-4 cc. amounts. When the second stage was fully established in the recipient, both animals were given 5 cc. each of heparin solution (10 mg. per cc.), and crossconnections were made with the cannulae by means of short rubber T tubes in such a way that blood flow from the proximal end of the carotid artery of each passed into the distal end of the other and so toward the brain, or, in the instances in which the jugular vein had been cannulated, from carotid artery to vein. Mercury manometers were attached to each of the tubes connecting the cannulae. These afforded continuous readings of the pressure at which blood flowed from one animal to the other. By clamping the tube on either side of the manometer by-pass, the blood pressure in the carotid of either animal could be obtained. The tubes leading to the manometers also carried a short side arm attached to a greased Lucr syringe through which blood could be added to or recovered from the circuit at will. Immediately before beginning cross-transfusion the blood pressure of the recipient was brought up to that of the donor, when it was somewhat lower, by the addition of as much compatible defibrinated blood as necessary. The blood, already taken from one of the donors, was given through a syringe to the donor of the cross transfusion and that animal was allowed to bleed an equal amount into a second syringe. This blood was in turn given to the recipient. In this way we avoided direct injection of defibrinated blood into the sick recipient, yet without changing the donor's blood pressure allowed the latter to furnish blood enough to the recipient to equalize the pressures in the animals. The cross-transfusion was then begun and the flow of blood adjusted thereafter by partial occlusion of the connecting tubes, so that the blood pressure of the liverless recipient remained throughout the experiment at approximately the same level as previously, or very slightly higher.

It was found by repeated determinations in the majority of experiments that the blood pressure of each animal at the proximal end of the carotid artery averaged about 5 mm. of mercury more than the transfusion pressure, that is to say the reading of the manometer without obstruction to the blood flow between animals.

The cross-transfusions were allowed to run for 2-4½ hours. At frequent intervals tests for flow in the connecting tubes were made by means of the greased Luer syringes. Simultaneous obstruction of the afferent blood flow to each animal close to the distal carotid (or jugular vein) cannulae caused the efferent streams of blood to pass into the syringes. By releasing the obstruction and in turn preventing efferent flow from both animals the material in the syringes could be expelled by way of the distal cannulae. In the instances in which jugular cannulations were used the jugular veins at each end of the venous cannula showed a bulbous swelling which disappeared at once when the tubes leading to them were pinched. Furthermore blood flow was usually visible in the cannulae.

As the transfusions continued the blood pressures of donor and recipient usually fell slightly. At intervals of approximately 20 minutes,  $2\frac{1}{2}$ —4 cc. of a 1 per cent heparin solution was injected in small doses into the tube leading to the donor. Transient falls in blood pressure resulted but were recovered from rapidly. Occasionally the blood pressure of one animal or the other would increase and there must have resulted some surge of blood from the one with higher pressure to the other. But pressure equilibrium was always soon re-established.

In two of the experiments the untouched carotid artery of the donor rabbit was ligated after the cross-transfusion had run for half an hour. This rendered the cerebral circulation of the donor almost wholly dependent upon blood supplied by the liverless rabbit.

As in the "liver transfusions" so in these the cross-flow of blood was terminated, when desired, by cutting and plugging the connecting tubes. Thus all traction on cervical nerves was avoided. As soon as a cross-transfusion was discontinued the liverless animal was given glucose, its corneal and musculo-motor reflexes were tested and it was examined for ataxia.

In the twelve experiments, five of which were wholly free from technical errors, the liverless rabbits showed no improvement. During the cross-transfusions the pulse rate slowed slightly, as one would expect with the advancing second stage of liver insufficiency. Immediately after the transfusions the animals showed no amelioration of the symptoms of the second stage. The muscular weakness, ataxia, apparent blindness and diminution of the corneal reflexes were usually more pronounced than before the transfusion. In a few instances gasping respiration, present before the transfusion, ceased for the first half hour of blood flow but began again before the transfusion

was finished. In general it can be said that the second stage of hepatic insufficiency progressed during the cross-transfusion itself, although its progress was usually far more rapid after the procedure.

In three of the five experiments the liverless animals entered the second stage with normal or almost normal blood pressure. In two instances abnormally low pressures existing an hour or two before cross-transfusion were rectified by injections of blood and further raised when the cross-transfusion was begun. The improvement was slight and short lived, however, and the phenomena of the second stage of liver insufficiency became more marked during the transfusion period,—this despite the fact that the pale skin of these recipients often became pink and that in one instance the blood pressure was raised from 40 mg. of mercury to 100. Clearly the late course of events after liver deprivation, that which leads to death, is not averted or even importantly delayed by cross-transfusion. We have rigorously ruled out all instances in which slips in technique or pathological conditions might have affected the results.

#### DISCUSSION

The work here detailed has shown the late changes occurring in liver insufficiency to be irreversible. What can one say of the origin of these lethal changes arising in the animal deprived of the liver?

It is evident that the second stage of liver insufficiency does not result merely from an overwhelming accumulation of toxic substances in the organism, which might be eliminated or neutralized by a liver if one were supplied. It is known that the products of incomplete metabolism accumulate in the blood of the liverless animal. Bollman, Mann and Magath (11) have reported the presence of increased amounts of uric acid and a probable increase in ammonia (12) in the blood of dogs deprived of the liver. Work already reported from this laboratory has confirmed these uric acid findings in rabbits suffering from pronounced liver insufficiency (6). Doubtless other products of incomplete metabolism accumulate in the blood of the animals as well. Both in the "liver transfusion" and cross-transfusion experiments there was ample opportunity for the donor liver to destroy or excrete toxic substances in the blood if such had been present. Furthermore in the cross-transfusion experiments there must have oc-

curred a dilution of any toxic substances, present in the recipient's blood, by admixture with the donor's blood. Despite these favorable conditions no improvement was observed in the condition of the liverless animals.

A toxic product accumulating in the blood of a liverless rabbit in amounts sufficient to kill might be expected to register some deleterious effect upon a normal animal through which the entire blood bulk, of the liverless one was suddenly circulated by transfusion. But no such effect was observed upon the donor rabbits in our cross-transfusion experiments. The animals serving as donors were allowed to recover from the anesthetic and carefully observed. They exhibited no abnormal symptoms whatever, even in the instances in which the hepatectomized rabbit's blood was allowed to run cephalad into the carotid artery of the donor, thus coursing through the brain and central nervous system of the latter before reaching the liver.

The symptoms of the second stage of liver insufficiency cannot be due to the mere lack of some essential substance, hormone or what not, which can be supplied from without at a late stage, as can sugar to prevent early death from deprivation of the liver. Previous work on hepatectomized animals (1-9) has not demonstrated a lack of essential substances other than glucose, but such work has not sufficed to exclude the possibility. The present work has made plain the fact that the normal organism cannot supply anything to the liverless one that will do away with or even ameliorate the second stage phenomena after hepatectomy, once these have appeared. the second stage had failed to appear until the lack became so great that irremediable damage had been done. Against the validity of this improbable assumption there is evidence, but unfortunately no proof. The liverless animal at the time of the onset of the second stage is normal in appearance and our histological examinations at autopsy, even in the untreated liverless animal, show no definite degenerative changes in the central nervous system or elsewhere. Yet cross-transfusion fails to prolong life in these animals. The outlook for prolonging the life of human beings with severe liver insufficiency by supplying "biologicals" is not a promising one.

An accumulation of toxic substances or a lack of something essential there may be in the hepatectomized organism but if so this lack or accumulation has resulted in irreversibly damaging changes by the time the second stage has set in.

Was there an accumulation of toxic products which became fixed upon the tissues? The foregoing discussion has concerned itself chiefly with the possible presence or lack of substances freely circulating in the blood of the liverless animals. A toxic substance present in the blood in minute quantity might conceivably be continually removed or rendered harmless by the liver, but become fixed within the tissues on the absence of the organ, causing irreversible damage. Such a happening would account for the failure of cross-transfusion to cause any betterment. An irreversibility of the changes of liver deficiency as manifested by the second stage phenomena has been the invariable finding in our work.

Yet another possibility remains. Perhaps the liver by means of minute amounts of some substances controls the activity of other organs, and when it is lacking, some irreversible change takes place.

#### SUMMARY

The symptoms of advanced liver insufficiency, in the hepatectomized rabbit, are irreversible. That is to say, cross-transfusions between liverless rabbits and normal ones fail to ameliorate the symptoms of liver deprivation once they are established. The normal rabbits show no symptoms suggesting that toxic substances have accumulated in the blood of the hepatectomized ones.

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### BIOLOGICAL STUDIES OF THE TUBERCLE BACILLUS

III. Dissociation and Pathogenicity of the R and S Variants of the Human Tubercle Bacillus  $(H_{37})^*$ 

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PLATES 31 TO 33

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One of the most vexatious occurrences in experimental tuberculosis is the lack of uniformity of infection and the variability in the extent of the disease in animals inoculated with tubercle bacilli. Two main factors have been offered in explaining this irregularity; namely, (a) the natural resistance of the host and (b) the virulence of the organism.

The first factor, which plays an important part in infection, is much more difficult to discuss for the reason that our knowledge of natural resistance is very meager.

In the past the virulence of the organism has often been masked by the large dosages used for experimental purposes, and this probably has been the main reason for a complete failure in an apparently promising experiment. Again, when a smaller dosage has been used for infection, the lack of uniformity of the extent of disease has been rather discouraging. This irregularity of disease has been mainly attributed to the virulence of the organism, an observation to which we agree. But why such a variation in an apparently virulent culture? Let us for a moment discuss the probabilities which very likely may influence virulence.

An ordinary culture cultivated at 37.5°C. represents a large population of tubercle bacilli propagating in normal conditions necessary for existence. It is not conceivable that every member of this population possesses the same degree of virulence for experimental animals. If we accept this hypothesis as a basis for discussion, then it follows that the virulence of the culture will depend largely on the ratio of the

<sup>\*</sup> This work was supported by a grant from the Rockefeller Foundation.

virulent to the non-virulent members of the culture. If we are correct in this assumption, then we can readily explain why cultures propagated in ordinary (up to the present uncontrolled) environments, show diversities in virulence. The virulent, as well as the non-virulent individual bacilli, are very sensitive to environments which can stimulate or retard their propagation. They probably possess some inherent characteristics which will manifest themselves in culture media. Visualizing all that may take place in cultures, it seems that, by assortment and elimination, in time we should be able not only to separate the two types, but also to stabilize their virulent and non-virulent characteristics by cultivating them in suitable environments.

Microbic dissociation studies, which have been in progress in this laboratory for the last 8 years, have not only added a great deal to our knowledge of the variability and instability of the tubercle bacillus, but also have put in our hands a method to control the virulence of the organisms.

The studies which are about to be reported deal mainly with dissociation and the virulence of the human tubercle bacillus.

### EXPERIMENTAL DATA

A human tubercle bacillus, H<sub>37</sub>, which for the last 29 years has been extensively used in experimental tuberculosis and for the preparation of tuberculin, was used in this study. The selection of the organism was very important because the past history of its behavior could be correlated with what might happen when it was subjected to a forced dissociation. The organism, since isolation, has been cultivated on egg, beef broth, agar, and Proskauer and Beck's synthetic medium, containing from 3 per cent to 5 per cent glycerol. At times when cultivated on glycerol beef broth, a diminution of virulence has been reported by a number of investigators. However, when cultivated on a heavily buffered synthetic medium no diminution of virulence has occurred. These observations naturally suggested the influence of environmental conditions in developing the types of organisms we desired. An attempt was made to increase the virulent population by prolonged cultivation of the organism on synthetic medium, and for the non-virulent members the use of beef broth was suggested. This procedure, however, failed to bring about the desired result, because the cultures always contained the two types of organisms. Therefore, we resorted to the plate method of cultivation where, by selection and assortment and further cultivation on plate media, single colonies could be isolated in pure state for further studies.

<sup>&</sup>lt;sup>1</sup> Isolated by Dr. E. R. Baldwin in 1905 from the sputum of a patient, age 19, suffering from chronic pulmonary tuberculosis.

Dissociation technique as previously described and successfully used in the dissociation of avian, bovine, and B.C.G. cultures of tubercle bacilli was applied in this study (1).

Two years ago Petrofi and Steenken dissociated the H<sub>37</sub> culture, but the variants isolated at that time were not stable. The supposed R variant, although much less virulent than the S,² still had some tuberculogenic properties, and was not devoid of virulence. A number of efforts to repeat these early observations failed.

The problem was then approached from a different point of view. Instead of using only the ordinary glycerol beef broth medium of pH 7.2, which becomes acid as the growth accumulates and thereby brings about spontaneous dissociation, our efforts were concentrated on forced dissociation. By forced dissociation we mean the amplification of the factors which in the past had, in part, been responsible for the decrease of virulence. Two methods were applied: (1) Cultivating the organism on glycerol potato bile medium (Calmette (2)), a medium which has been successfully used for diminishing virulence. (2) Subsequent cultivation for assortment and elimination, and, especially where the R, avirulent variant was desired, cultivation on solid and fluid media of pH 6.1. The influence of pH on the dissociation phenomenon (3), and a method for pH determination of solid media (4), have been described by one of us.

Media Used .- The following media were used in these experiments.

Solid medium: Gentian violet egg medium of pH 6.1 and 7.2; glycerol beef agar; Calmette's glycerol potato bile medium.

Fluid media: Beef broth; potato broth; and Proskauer and Beck's synthetic medium, all containing 5 per cent glycerol.

Petri dishes and a special modified Kolle flat flask (5) about 10 cm. in diameter were used. As the tubercle bacilli, apart from their varied and long lag periods, propagate very slowly, special precautions were taken to eliminate air contaminants as much as possible. Flasks proved more satisfactory for this purpose than plates. After seeding, and being plugged with cotton, the flasks were capped with rubber nipples having at the tip three small holes for gaseous exchange. This we considered one of the most important steps in the technique (6). Repeatedly we had noticed that if the cultures were deprived of atmospheric oxygen by tightly scaling the mouth of the flask with melted paraffin, not only was the growth retarded, but the topography of the individual colonies was so modified that a selection was almost impossible.

### First Dissociation

Several tubes of Calmette's glycerol potato beef bile medium were seeded with growth from a stock synthetic culture and incubated undisturbed at 37.5°C. for 9 months. At the end of that time small portions of the growth were triturated against the side walls of a small test tube and a suspension made in synthetic media.

The symbol R has been used previously in referring to the variant which is resistant, and S to the variant which is sensitive, to environment.

The suspension was filtered through Whatman paper No. 5 and 5 drops of the filtrate seeded on the surface of several gentian violet egg medium flasks of pH 7.2 for single colony study.

Minute single colonies appeared at the 3rd week. At the 5th week, they had increased in size to the stage at which their structure could be studied. Two distinct and many atypical colonies could be recognized. One was made up mainly of twisted, wormy, slightly moist coils with a clean-cut periphery and was labeled R? variant (Fig. 7, a). The second colony was slightly raised, stippled, and had a veil-like appearance, and this was designated S? type (Fig. 7, b). Suspensions in saline of these two colonies were prepared and after the concentration was determined by dry weight (7), dilutions were made so that each cubic centimeter contained approximately 5,000,000 organisms.

Four guinea pigs were then inoculated intratesticularly with 5,000,000 organisms each of the R? variant and four with the same dose of the S? variant. Two of the R? animals died of tuberculosis (70 and 110 days); one was killed at 102 days and one died of pneumonia at 105 days. The gross examination showed considerable tuberculosis, especially in the first and last animals.

An animal of the S? group died of tuberculosis at 102 days, and the other three were killed at 70, 105, and 110 days. In general, they had more tuberculous involvement than did the R? animals and there were more bacilli present in the organs. The differences, however, were not extreme nor surprising. (Sections from the second and third animals of each group were taken for histological study and will be described later.)

The necropsy findings thus indicated that the R? variant was not devoid of virulence. The R? colony was then subinoculated on potato bile medium, and at the same time suspensions prepared for and seeded on flasks of gentian violet egg medium with a pH of 6.1 and 7.2. 4 weeks later in the flasks inoculated with the R? suspension, many R? and S? colonies had developed. The two types of colonies again were fished out, suspensions prepared, and seeded again on gentian violet egg medium of pH 6.1 and 7.2. After a month of incubation in the flask of pH 6.1, seeded with S? suspension, atypical colonies had developed (Fig. 7). On the medium of pH 7.2, the colonies were typical S? type (Figs. 3 and 4). But the flasks which were inoculated with the R? suspension showed some very striking new types of colonies with a clear-cut periphery, a vermicular contour, crater-like center, and a slight amount of chromogenicity (Figs. 1 and 2). This method of assortment was repeated several times, and similar R? colonies eventually were obtained. R? colonies were also obtained directly from the potato bile cultures, and they were slightly more chromogenic than the aforementioned.

Ten animals were inoculated intratesticularly with approximately 100,000 bacilli of the S? variant and another lot of ten with the same number of the supposedly R?, crater-like, variant. In the R? group, one animal died of suffocation at 59 days; the remainder were killed at 8, 36, 36, 41, 63, 82, 96, 98, and 98 days. In the gross, one animal at 36 days had small lesions of the lungs, liver, spleen, and

inguinal glands; the one at 41 days small lesions of the lungs and liver; and at 63 days there were tiny lung lesions. The other animals exhibited only small lesions at the site of inoculation. Smears of tissues at necropsy, except for the testicle and the femoral bone marrow of the diseased 36 day pig, were negative.

Animals of the S? group were killed at 8, 59, 82, and 96 days. The other six died of tuberculosis at 36 (two), 41 (two), 46, and 63 days. The 8 day animal exhibited only a swollen testicle. The other nine were listed as having four plus tuberculosis, the maximal involvement. Every organ was extensively involved. The lungs contained discrete or conglomerate gelatinous or gelatinocaseous tubercles and areas of pneumonia. The livers contained both gelatinous tubercles and areas of necrosis and infarction, the least involvement being after 60 days. The spleens were enlarged from four to ten times normal size, the largest and most engorged being at 4 to 6 weeks, and often contained areas of necrosis. Every bone marrow appeared hyperplastic. Smears of the spleen and marrow at 8 days were negative for tubercle bacilli. The organs of the other nine animals were positive except for the marrow at 82 days. The largest number of bacilli occurred between 4 and 6 weeks.

The contrast between the pathogenicity of these R and S variants is obviously quite marked.

The R variant with a crater-like appearance on the fourth cultivation remained true to form, provided media of pH 6.1 were used. The virulence test was repeated by inoculation of four animals. Each guinea pig was inoculated intratesticularly with 200,000 of the R type bacilli.

An animal died of pneumonia at 34 days and others were killed at 24 and 65 days. The fourth was skin-tested at 370 days (negative) and killed. An inguinal lymph node at 24 days was enlarged and the site of inoculation in the 65 day animal contained a small scar. All other organs and sites were negative in the gross for tuberculosis. None of the smears or sections contained acid-fast bacilli. A further decrease in pathogenicity was evident.

## Characteristics of the Variants

Fluid Media.—Typical R and S variants, the virulence of which had been determined by animal inoculation, were cultivated on glycerol beef broth and synthetic media. The S variant in these two media developed a thin, spreading, veil-like growth, with only a few dense areas. The R variant in both media grew in small, raised islands, with clear spaces between, and only occasional net-like connections between. (Fig. 8—S variant; Fig. 9—R variant.)

Morphology of the Variants.—Both R and S variants are Gram-positive and acid-fast. The S bacilli are short, solid rods, sometimes slightly curved, varying in length from 2 to 5 microns. Beaded forms

are only occasionally present and granules are absent (Fig. 5). The R bacilli are pleomorphic, varying in size from 3 to 10 microns. Some are club-shaped, others straight, filamentous, long, beaded rods, and still others appear in branching form. Granules of various sizes are present in very young cultures. Many non-acid-fast organisms are present in young cultures but as they become older they gradually regain their acid-fast properties (Fig. 6).

Reaction of Media.—The pH curve of the medium during growth of the tubercle bacillus has been extensively used for differentiation of the human and bovine types. Since the S variant is more stable in an alkaline medium, and the R in an acid medium, a study of the pH of the medium during growth was thought to be of interest. The two variants were cultivated on three types of broth with two different pH levels each. Electrometric determinations were made at weekly intervals for 7 weeks. Starting with a pH of 6.8, the media on which the two variants were cultivated became alkaline (pH 7.5) within a week but, as the growth progressed, a depression in the curve was noted, and from the 2nd to the 4th week, the exact time depending on the rapidity of the growth, it became strongly acid (pH 5.5).

From our observations thus far it is safe to state that the pH curve of both variants follows that observed for any undissociated human type of tubercle bacillus.

Tuberculins.—Old tuberculin was prepared from the two variants using glycerol beef and synthetic broth cultures. The tuberculins were tested intracutaneously on guinea pigs sensitized with heat-killed R and S variants. Both tuberculins elicited the same type and intensity of reaction. However, the antigenic properties of the heat-killed organism used for sensitization differed. Animals injected with heat-killed S variants developed a hypersensitive state sooner and with greater intensity than those sensitized with heat-killed R variants.

### Second Dissociation

In the preceding experiment we may recall that the initial culture taken from synthetic media was undissociated and the components were unknown. Naturally, this successful dissociation suggested a further experiment, the starting point of which would be a single definite colony. An S colony with typical cultural characteristics and virulence for guinea pigs was seeded on glycerol potato bile medium and incubated undisturbed at 37.5°C. for 4½ months. A suspension was prepared from this aged culture and seeded as usual on gentian violet egg medium, glycerolated agar of pH 6.1 and 7.2. 1 month later typical and atypical S colonies, and a large number of crater-like R colonies were distinguishable. At this time exceptional growth was noted on gentian violet egg media at pH 6.1; fair growth on glycerolated agar at pH 6.1; and a slow growth on both at pH 7.2. Subculture of the R and S variants was continued until the two types were further stabilized, and at intervals animals were inoculated to test the virulences.

Eight guinea pigs were first inoculated with the R type,—four with the culture from glycerolated agar pH 6.1, four from gentian violet egg medium pH 6.1; and four were inoculated with the S type from gentian violet egg medium, pH 7.2. The infecting dose was 500,000 bacilli administered intratesticularly.

Two of the R animals were killed at 31 days, two at 38 days, and one at 97 days, while two died of gastro-enteritis at 45 days and the last of pneumonia at 104 days. The only gross tuberculosis in the series is found in the form of tiny lesions in the liver and inguinal nodes of a 31 day pig. Routine smears of all animals were negative for tubercle bacilli.

S-infected animals were killed at 31, 38, and 45 days and one died of tuberculosis at 97 days. All of the four were noted in the gross as having four plus tuberculosis, there being marked involvement of the lungs, liver, spleen, inguinal nodes, and testicular site of infection. Swelling and necrosis of the spleen, and necrosis in the liver were as described for a previous group.

Two weeks later variants from cultures of the same series were used to infect rabbits, two being infected with the R type and three with the S type. 1 mg. was given intravenously to each.

One R animal was killed at 27 days and the other at 32 days. Neither had gross tuberculosis and marrow smears were all negative for acid-fast organisms. One of the S group died of apparently acute tuberculosis on the 27th day, and two were killed at 32 days. In the gross, the lungs of each contained many single and conglomerate gelatinocaseous areas of tubercle and pneumonia. There were pale streaks in the kidneys. The femoral bone marrows were negative by smear but the lungs, kidneys, and a liver and spleen contained bacilli.

In this first test on rabbits there was the same marked difference in pathogenicity noted for guinea pigs with the modifying effect of species evident.

A final test of the virulence of variants from the first complete dissociation was made at this time in order to study tissue reactions with the supravital technique. 0.5 cc. of a milky suspension of bacilli was inoculated intraperitoneally into guinea pigs. The R variant came from a subculture (the fourth) on gentian violet egg medium at pH 6.1, and was used on three animals. Three other animals were inoculated with an S variant from this medium at pH 7.2.

An animal from each group was killed on the 1st, 2nd, and 7th day after infection. There was no marked difference in the peritoneal fluids. The site of inoculation was slightly necrotic in animals of both groups. The omentum of the R animals showed a slight stimulation of the "milk spots" throughout, but in each case it was less than in the contrasting S animal. There was also a progressively increasing tendency to small areas of necrosis in the S omentums, but none in the R. Tubercle bacilli were rarely found in the peritoneal fluid and omentum of the R inoculants, but were present in large numbers at all times in the S series. (The supravital studies are discussed later with the microscopic pathology.)

Further inoculations to determine the pathogenicity of the variants from the second dissociation, using different portals of entry, were begun after a 4 months' interval.

Guinea pigs were infected by subcultures of the R variant from gentian violet egg medium at pH 6.1, and of the S variant from the same medium at pH 7.2. Five animals received 10,000 each and five received 100,000 each of the R type bacillus subcutaneously. Contrast groups were given the same doses of the S suspension by the same route.

One of the R (10,000 bacilli) pigs died of pneumonia at 25 days; the others were killed at 52, 59, 145 days (two). No gross evidence of tuberculosis was present in any animal. Smears of the spleen, regional nodes, and bone marrow were negative for acid-fast organisms in all cases.

The S (10,000 bacilli) infected animals were killed at 15, 25, 52, 59, and 145 days. No organic disease was seen in the first two but the regional nodes were enlarged and contained many bacilli. At 52 days there was considerable disease of the lungs, liver, and spleen; this distribution was duplicated at 59 days except for the absence of lung involvement; and the identical organs were tuberculous at 145 days. In the later animals the inguinal, iliac, and tracheobronchial lymph nodes became larger, harder, and more caseous. Bacilli were not found in the marrows, but the organs often were positive.

One of the second R group (100,000 bacilli) died of pneumonia at 15 days. Others were killed at 38, 59, 130, and 145 days. Four animals were normal except for slight inguinal node hypertrophy in the first two. The spleen and a lymph node in the 130 day pig contained possible tiny lesions, but smears were negative for tubercle bacilli, as they were in all of the other animals.

One of the second S group (100,000 bacilli) died of tuberculosis at 84 days; the others were killed at 39, 59, 130, and 145 days. The amount of tuberculosis did not increase regularly with the duration of the disease. At 39 days there were

small lesions in the spleen, lungs, liver, and inguinal nodes. At 59 days only the liver and nodes were involved. In the animal that died at 84 days a marked disease of the spleen, lungs, and liver was seen and the 130 day animal was approximately the same. There were areas of necrosis in the livers, and the spleens were four times normal in size. At 145 days there was less involvement of the spleen and lungs. The later lymph nodes showed the same increase as in the previous S group, and smears were positive for bacilli. The marrows were negative and only the spleens at 39 and 145 days were positive.

After an interval of a month and a half another generation of subcultures was used to infect rabbits and guinea pigs.

Typical R colonies from gentian violet egg medium at pH 6.1, and S colonies from the same medium at pH 7.2 were used for both species. Five rabbits received 1 mg. each of the R variant intravenously and five received 1 mg. of the S type.

Individuals of the R group were killed at 7, 20, and 82 days and one died of snuffles-pneumonia on the 82nd day. Tiny gelatinous lesions were seen in the lungs at 7 days, and smears showed a few tubercle bacilli, but all of the organs of the other animals were normal, and in only the spleen at 20 days were bacilli found.

One of the S rabbits was killed at 7 and one at 20 days; the others died at 27 and 36 days of snuffles-pneumonia. At 7 days there were small lung and spleen lesions and all organs contained bacilli. In the other animals the lesions were larger and chiefly pulmonary. The latter were gelatinocaseous, single and confluent, and contained many bacilli. The liver, spleen, and kidneys were to a lesser extent irregularly involved, and usually contained bacilli. The marrows were positive except in the last animal.

The guinea pigs were infected intratesticularly with 5,000,000 organisms from the suspensions used above. The R and the S variants were each used in four. This experiment repeated the first comparison which was made with the intermediate dissociates.

One of the R group was killed at 30, at 60, and at 106 days. One died of streptococcus abscesses at 91 days. At 30 days, the inguinal glands and spleen were slightly swollen, and a few bacilli were found in the smears of these tissues. In the other three animals none of the viscera contained gross lesions, no bacilli could be found, and the testicular sites showed only slight localized congestion and, in one case, atrophy.

One of the S group was killed at 30 and at 60 days; one died of tuberculosis at 64 and at 95 days. All four were listed as having four plus involvement. The lungs were riddled with tubercles, at first small and grey, later confluent and caseous. The spleens were enlarged to 4, 8, 10, and 12 times the normal size and showed increasing loss of structure, hemorrhage, fibrin, and necrosis. They all contained many tubercle bacilli. The livers were markedly involved with areas of necrosis in the last two. The marrows at 60 and 64 days contained tubercles and bacilli. The testicles were caseopurulent and had lost structure. The inguinal, iliac, and tracheobronchial glands were regularly enlarged and contained bacilli.

Again the contrast in pathogenicity is seen to be very marked between the two variant groups.

One month after the inoculations of the previous series, subcultures of a following generation were used to infect rabbits by the infrequently used intratesticular and subcutaneous routes.

The same dose previously used intravenously, 1 mg., was given to three animals for each variant and for each route. The R colonies came from gentian violet egg medium at pH 6.1, and the S colonies from the same medium at pH 7.2.

The R intratesticular animals were killed at 37 and 89 days (two). There was no gross evidence of tuberculosis except a small area of caseation at the site of injection in the second animal. Smears of the spleen, lung, and marrow were negative in each case. (Two animals showed localized chronic bronchitis and pneumonitis.)

In the contrasting S group an animal died of four plus tuberculosis at 19 and one at 37 days. The third died of snuffles-pneumonia and contained a moderate tuberculous involvement. The lungs of the first two were riddled with gelatinous tubercles, the testicles were swollen, purulent, and contained bilateral miliary lesions, the kidneys were streaked, and the follicles of the spleen were accentuated. In the third animal the lesions of the lung were smaller but there were miliary lesions of the omentum and spleen. Testicular smears were loaded with tubercle bacilli, the lungs were positive at 19 and 37 days, and bacilli were found in the spleen of the first.

An R subcutaneous animal died of snuffles at 23 days and others were killed at 51 and 89 days. There were no lesions seen in the gross except small doughy foci at the sites of injection. All smears except from the inoculation site were negative.

In the S contrast group an animal was killed on the 23rd and 89th days and one died of snuffles-pneumonia at 51 days. Each had a moderate involvement of the lungs and caseous scarring at the sites of infection. Smears of the spleens and marrows were negative, but the lung at 23 days and the sites were positive for tubercle bacilli.

# Microscopic Pathology and Hematology

The routine pathological study included the following procedures and precautions:

(a) Animals were autopsied as soon as possible after death. Guinea pigs were killed by a blow at the base of the skull, rabbits by a blow or air embolus. Unless the groups were small or deaths too frequent in one variant group, an animal in the contrasting group was killed whenever one died.

- (b) The usual complete gross examination was made, including opening of the nasal and middle ear passages. The approximate amount of tuberculous involvement was noted (0 to 4+). Smears were made routinely from the femoral marrow, spleen, and questionable lesions. The site of injection or regional lymph nodes were examined when the viscera contained no bacilli. The smears were stained by the Ziehl-Neelsen method and an approximation of the bacillary content was noted (0 to 4+). Smears for secondary infection were studied with Gram stain, and when no obvious cause of death was present heart's blood cultures were made in pleural fluid broth.
- (c) Sections of the lungs, liver, spleen, kidney, and usually the bone marrow, lymph glands, and site of inoculation were taken, fixed in Zenker's 5 per cent acetic acid solution, run up into paraffin blocks, cut, and stained with Mallory's phloxinmethylene blue stain. Duplicate sections were stained with carbolfuchsin and methylene blue for identification of acid-fast organisms.
- (d) An attempt was made to study the reticulum formed in tubercles of the two types of infection, but as it is incomplete a detailed report is planned for future publication.
- (e) The cytology of the blood was observed during the course of experiments in six groups of animals.
- (f) Supravital studies were made on the tissues of nineteen animals from various groups.

The description of microscopic findings will be presented in the same order as that of the gross examinations.

- 1. R? (intermediate): 5,000,000 bacilli intratesticularly in four guinea pigs. Tissues from two animals were saved (102 and 105 days). The lesions were seen to be chiefly proliferative and to contain small areas of caseation. The spleens and livers were riddled with this type of infiltration and the lungs contained lesser amounts. The disease appeared to be chronic with occasional evidences of activity.
- S? (intermediate): Same procedure as above. The lesions were slightly more extensive in all organs. They were chiefly chronic proliferation with considerable activity, and the organic structure was therefore frequently destroyed. The epithelioid cell tubercles were often involved by cascation. Both spleens were acutely inflamed, with edema, congestion, and fibrin, but with relatively few bacilli. Acid-fast granules were seen in vacuolated phagocytes. An exudative and proliferative pneumonia was present in one lung section.
- 2. R: 100,000 bacilli intratesticularly in ten guinea pigs. In the animals sacrificed between 36 and 63 days there was found small to moderately extensive tubercle formation of a sluggishly proliferative type. The cells were moderately stimulated members of the mononuclear series and they were usually circumscribed by flattened cells and lymphocytes. Giant cells of the Langhans type were occasionally seen but cascation never occurred organically. At its height the in-

fection involved the spleen and liver (Fig. 11) to about the same extent. Regression began as early as 36 days in the liver; and after 63 days, when any lesion was present at all, only small, resolving, or completely regressive lesions were found in the organs of any animal. Healing was accomplished by a decrease in size and a pyknosis of the stimulated cells with a further peripheral contraction. Lung lesions were exceptionally difficult to find. They occurred interstitially and rarely grew to fill alveoli (Fig. 10). There was no distinctive feature with regard to the size or position of the lesions in any organ, except the regressive tendency, and no evidence to indicate extension or chronicity. A search of all of the acid-fast sections revealed a single bacillus in the spleen at 63 and at 82 days.

S: 100,000 bacilli intratesticularly in ten guinea pigs. Tubercle formation had already begun at 8 days. The lung was considerably involved by large and small lesions of the alveolar walls, as well as a general stimulation of the parenchyma. The former were rapidly growing, as denoted by mitoses. The liver contained a few intersinusoidal groups of mononuclears.

The next six animals can be discussed as a group, for though there was some difference in acuity, the lesions of all were considered to be extremely progressive. The primary proliferative lesions, composed of mature epithelioid cells, contained central abscess formation (Fig. 12) and cascation, most commonly seen in the animals at 36 days. Tubercles in the walls of veins (Fig. 15) and abscess debris in the bronchioles (Fig. 14) were seen repeatedly. Fresh areas of exudative pneumonia were observed in the lungs, as were areas of infarction and areas of necrotic tissue cells contiguous to acute lesions in the liver (Fig. 16). There was a complete loss of structure of the splenic tissue, with replacement by serum, blood cells, and a network of fibrin. Tubercles were seen as isolated proliferative and caseous islands (Fig. 18). The bone marrows, referred to as hyperplastic in the gross, were preserved with difficulty. Those examined (4) showed a moderate involvement by loose epithelioid cell tubercles with occasional central degeneration and caseation. There was a hyperplasia of the neutrophile marrow cells.

Sections of the 63, 82, and 96 day animals showed widespread disease, but it was of a more chronic type (Fig. 19). There was less evidence of acute infection, abscess formation, and swelling. Part of the lesions in most organs were definitely regressive, and exhibited resolution and efforts at repair. The lungs contained the only notably progressive lesions. The process of resolution included a loosening of the epithelioid cells, pyknosis, emigration of cells from the tubercle with a serum-fibrin replacement, and the presence of many lymphocytes in and around the tubercles. Sections stained for bacilli confirmed the knowledge obtained from smears at necropsy: at 8 days only the testicle contained bacilli; from 4 to 6 weeks all organs, especially the lung and spleen, were heavily positive; at 3 months the organs, though riddled with disease, contained fewer bacilli. Their position, in order of frequency, was: phagocytic mononuclears, degenerating epithelioid cells, caseation, and debris.

3. R: 200,000 bacilli intratesticularly in four guinea pigs. Except in the testi-

cles, definite microscopic lesions were found only in the liver at 24 days and the lung at 65 days. These consisted of small proliferative lesions, non-progressive in type. The 370 day animal contained a few swirls of fibrosis which may have been tuberculosis near a large bronchiole. No bacilli were found in the sections of any animal.

- 4. R: 500,000 bacilli intratesticularly in eight guinea pigs. The involvement of this series was similar to that of the R in Series 2, but was smaller in extent. At 31 days a moderate proliferative tubercle formation was present. Tiny regressive lesions were seen in a spleen, and two livers of the next three animals killed. The last three pigs showed no disease (45, 97, and 104 days). No bacilli were found in the sections, and in a lymph gland at 31 days three bacilli were seen.
- S: 500,000 bacilli intratesticularly in four guinea pigs. Microscopic sections were practically duplicates of the S sections in Series 2. The lesions were composed of stimulated epithelioid cells, abscesses, acute caseation, and in certain organs tissue degeneration and necrosis with anemic infarction. There were many giant cells. At 97 days there was huge involvement but considerable resolution. The whole picture was slightly less acute than that of the 100,000 group, Series 2.
- 5. R: 1 mg, intravenously in two rabbits. There were small lesions in lungs, liver, spleens, and one marrow. They were completely proliferative, without necrosis, and were often composed entirely of giant cells (Fig. 13). The mononuclear cells were only slightly stimulated and the lesions were often surrounded by lymphocytes. They were all inactive or regressive. There were no bacilli seen in the sections.
- S: 1 mg. intravenously in three rabbits (sections of two). The lesions were large in extent, especially in the lungs. They were composed of infiltrating epithelioid and giant cells and areas of proliferative pneumonia (Fig. 17). Caseation was present centrally in the lesions of the lungs, a liver, and a kidney. No true abscess was seen. The tubercles were of a moderately progressive type. Bacilli were found in the sections of the lungs in large numbers, and in moderate numbers elsewhere.
  - 6. (Sections are discussed with the supravital studies of this group.)
- 7. R: 10,000 bacilli subcutaneously in five guinea pigs. There were no definite lesions in the sections examined. No tubercle bacilli were found.
- S: 10,000 bacilli subcutaneously in five guinea pigs. There were early proliferative lesions in the lungs and liver at 15 and 25 days. At 52 days the lesions were larger and beginning to degenerate and there were large lesions in the spleen with central abscess and cascation.
- 8. R: 100,000 bacilli subcutaneously in five guinea pigs. The extent of disease was limited to small groups of epithelioid cells in the spleen at 38 and 130 days and the liver at 59 days. These processes were inactive or regressive and no necrosis or bacilli were seen.
- S: 100,000 bacilli subcutaneously in five guinea pigs. The progress of infection was similar to other S infections already described. The lesions were proliferative and caseous at 6 weeks, extensive, and at later dates were acute and florid.

The progression of infection was definite and the type of disease was the same at both doses subcutaneously as it was in the groups infected intratesticularly, but the organic involvement was less regular and the advance was delayed.

- 9. R: 5,000,000 bacilli intratesticularly in four guinea pigs. Sections of the spleen at 30 days contained a few small proliferations in the follicles, slightly caseated, and a small area of epithelioid cells in the marrow. No bacilli were seen. There were no lesions seen in the later animals.
- S: 5,000,000 bacilli intratesticularly in four guinea pigs. This series exhibited the same rapid extensive involvement, with abscess formation, caseation, and secondary inflammatory signs as other S groups, but even more intensely. Tubercle bacilli were seen as thin, almost bipolar, poorly staining rods and were found in great numbers in the debris, serum, and marginal epithelioid cells and phagocytes.
- 10. R: 1 mg. of bacilli intravenously in four rabbits. A few small proliferative lesions were seen in the first animal, chiefly in the lungs. A tiny group of loose epithelioid cells was present in the spleen at 20 days. At 80 and 82 days there were no lesions.
- S: 1 mg. of bacilli intravenously in four rabbits. Progressive lesions, even at 7 days, were seen in the lungs. They were acutely proliferative with abscess and caseation. The livers, spleens, marrows, and kidneys contained proliferative lesions which were caseous in the kidneys and, in the later animals, regressive in the liver. A considerable number of giant cells was present.
- 11. R: 1 mg. of bacilli intratesticularly in three rabbits. Small inactive proliferative tubercles were present in the lung, liver, and testicle at 37 days. The lesions in the lung consisted of loose epithelioid cells in the dilated blood vessels of an old snuffles-pneumonia.
- S: The lungs, spleens, and testicles and, less regularly, the liver and bone marrow contained large numbers of proliferative lesions. Caseation occurred in the lungs and testicles. All tubercles appeared progressive.
- 12. R: 1 mg. of bacilli subcutaneously in three rabbits. A single group of loose epithelioid cells in a splenic follicle was the only lesion seen.
- S: The lung and liver at 23 days contained discrete proliferative tubercles. Later sections were incomplete.

(Animals of the last three groups were often snuffly and the sections contained several areas of chronic bronchitis and indurative pneumonitis, small intersinusoidal nuclear groups in the liver, and similar interstitial areas of chronic inflammation in the kidneys. These have been previously described, and we have found them in other rabbits with snuffles.)

# Hematology

*Procedure.*—Blood studies were made on the variant groups of two series of guinea pigs (1, 4), and one series of rabbits (10).

Three consecutive daily counts were made on every uninfected animal during the week before infection. Counts were made after infection on guinea pigs at weekly intervals, considered frequent enough because the route of infection was intratesticular. Counts were made on rabbits (intravenously infected) at 24 hours after infection, at 48 hours, 6 days, and weekly thereafter. From previous experience, and from reference to studies on the cellular changes in the tissues after intravenous infection (8), it was believed that these dates would demonstrate the blood changes most effectively.

Guinea pigs were bled from a cut in the ear margin. Total white blood cell counts were made with the usual technique; fixed smears were made on cover-slips and stained with Wright's stain. Rabbits were bled from a nick in an ear vein, and total red and white blood cell counts, clotting times, and fixed smears were prepared. The percentages of cells in the differential count (200 to 400 cells) were used to compute the cells per cubic millimeter. A base line for each cell type was obtained for each individual by averaging the figures of the three control counts.

The composite graphs for each group were obtained by averaging the figures of the component members. The base lines, for comparison of the curves with the normal cell levels, were obtained by using the average of the individual base lines.

There are several factors which influence the interpretation of individual and group graphs of leucocyte counts. (a) Rabbits are prone to chronic secondary infection, especially snuffles and thrush. One group studied here (R, Series 10) unfortunately was affected by both during the latter part of the study. Guinea pigs may have chronic aural or nasal infections, but no animal in our series presented these findings at necropsy. (b) Individual animals may have unusual normal cell levels which cause a regular distortion of the curve until the size of the group is decreased by death. (c) This factor of decreasing units then causes the unusual cell level to distort the curve unequally. (d) Although it is known that several animals will react similarly, in general, to the same infection, it is also true that the time of the reactions will be slightly different in the individuals. This has been borne out by recent studies in this laboratory of infection by avian dissociates in rabbits (unpublished).

The pathological background which is used for the interpretation of abnormal changes in the curves is that postulated by Medlar (9). It includes the function of the monocyte in tubercle formation, the lymphocyte in the healing process and as an evidence of resistance, and the neutrophile in abscess formation and in cascation. The graph

of blood changes in a rabbit infected with bovine bacilli intravenously, first cited by Sabin some years ago (10), expresses the general reaction of an animal to pathogenic tubercle bacilli. We believe (from the avian work mentioned above) that all changes in the curves depend for their intensity on the virulence of the organism because the virulence dictates the pathological picture. A virulent variant causes a rapidly progressive disease with necrosis, abscess formation, and caseation (especially in the guinea pig), and a consequent intense blood reaction with an emphasis on the neutrophile. An avirulent variant causes a proliferative, sluggish, resolving infection which, if not complicated, causes a less intense reaction and a return of the blood curves to normal. We have found no evidence of a relationship between tuberculosis of the marrow and the depression of the marrow cells which occurs between the 2nd and 5th weeks. We have found evidence of toxic effects on the marrow cells (after intravenous inoculation) which appeared during this period (see Series 10 analysis).

Sections of the bone marrow were available from twenty-two of the thirty animals on which the blood was studied. A correlation was attempted.

Analysis.—Series 2 (R): There was a rise at 1 week of all cell types, a decrease of all types except the monocyte during the succeeding 4 to 5 weeks, and a return of all cell types to approximate normal a few weeks later. This would indicate a regressive infection. (Individual counts did not affect the curves materially.)

Series 4 (R): The neutrophiles and monocytes were slightly elevated at a week; the lymphocytes began a gradual depression which lasted for several weeks. A rise of all cell types, especially neutrophiles, after 7 weeks caused a distortion partially due to the consistent high figures of a single animal. The graph otherwise could indicate inactivity of the disease.

Series 2 (S): An elevation of neutrophiles at 1 week and again at 8 and 9 weeks occurred. There was a mild general depression from the 3rd to the 6th week. A terminal rise of the curves was due to the high counts of one animal; all other animals had shown a marked lymphocyte decrease before death. This interpretation would indicate a continuing infection.

Series 4 (S): All cell types increased at 1 week, the neutrophiles continued to rise at the 2nd, and they then joined the lymphocytes in a continued depression. The monocytes were slightly but increasingly elevated throughout. (The counts of the last 6 weeks were on only one animal.) The graph might indicate a continuing infection.

Series 10 (R rabbits): A depression of the leucocytes of marrow origin occurred

in the counts of the 1st week, with a rise of all leucocytes at 2 weeks, and a return to normal levels at 5 and 6 weeks. A moderate, constant depression of the erythrocytes was noted beginning at the 2nd week, but the microscopic structure of the cells was not affected. All changes after 6 weeks were unimportant because of the active secondary infections in both animals, a condition which the blood reflected. The graph could indicate a subsiding tuberculous infection.

Series 10 (S rabbits): The neutrophile curve failed to show a regular early rise, became depressed after 2 weeks, and rose at 5 weeks (one animal) to a high figure. The monocytes were increased for the first 3 weeks to relatively high levels. The lymphocyte curve rose gradually to a high level at 3 weeks and then fell to below normal. All of these curves were affected by unusual variations of individual counts.

There was a marked general depression of the erythrocyte curve beginning at 24 hours and extending through the life span of three animals. This deflection was caused by an erythropenia occurring in three of the animals, the low point occurring at 2 days in one, 2 weeks in another, and 3 weeks in the third. The lowest count reached 1,480,000. Differential smears demonstrated a marked anisocytosis, polychromatophilia, 5 to 28 normoblasts per 100 white blood cells, 5 to 24 per cent reticulocytes, and a shift to the left in the maturation of neutrophiles. A leucocytosis was associated in two cases; a leucopenia in one case. All red cell counts returned to normal before demise of the animals. The marrows were seen to be very slightly involved by a proliferative lesion at necropsy in two cases, and not at all in the most severe depression. Because of this and the time element, the reaction was possibly a toxic one and not disease of the marrow nor hemolysis.

Bone Marrow.—There was very little correlation between the degree of stimulation of the marrow cells and the position of the neutrophile curve at death. Most of those curves which were elevated at death were associated with a hyperplasia of granulocytes, but very frequently there was a hyperplasia, with a shift either to the right or left, and the curve was depressed or at a normal level. It was found that those with tuberculous involvement of the marrow had depressed or normal counts and those with elevated counts had no tuberculosis of the marrow, but on the other hand all those with tuberculosis had a hyperplasia of the marrow in addition.

Comment.—1. There are too many modifying factors to permit other than a loose interpretation of a composite graph.

- 2. In general, the blood count reflects the pathologic picture in the rabbit and guinea pig. It does not do so as accurately as in human beings. Individual animals react with different speeds and intensities to infection. Non-tuberculous infection causes a hopeless distortion of the curves.
  - 3. The R and S variants cause a leucocyte response which is par-

tially non-specific. This includes an initial rise of the marrow cells between 1 day to 2 weeks, and a depression occurring between 2 and 6 weeks. It is significant that the latter occurs approximately during the period of allergic development.

- 4. This experiment (and others in progress) shows that the avirulent variant (R) produces a regressive infection and blood curves which revert to normal. The virulent variant (S) produces a progressive, septic, allergic infection and the blood curves indicate these conditions to a certain extent.
- 5. The portal of entry modifies the periodicity and intensity of the leucocyte curves.
- 6. There is no notable relationship between the granulocytes in the circulation, the condition of the marrow at death, and the involvement of the marrow by tuberculosis.

# Supravital Tissue Examinations

The tissues of nineteen sacrificed animals were examined by means of the supravital staining technique.

Scrapings of two to five organs were examined, using the concentrated solutions of Janus green and neutral red which are prescribed for tissue study. The presence of abnormal numbers of neutrophiles, lymphocytes, or members of the mononuclear series (monocytes, clasmatocytes, and epithelioid cells) was noted, as was the condition of the stroma cells.

Tissues from animals infected with R variant (four rabbits, seven guinea pigs) were seen to contain mononuclear cells in moderate numbers and with a mild degree of stimulation. Neutrophiles were rarely seen and then only in glands near the site of inoculation. Tissues from animals infected with the S variant (four rabbits, four guinea pigs) contained large numbers of mononuclear cells in all stages, chiefly the mature epithelioid cells, and many neutrophiles as evidence of caseation.

Series 6 consisted of three guinea pigs inoculated intraperitoneally with 0.5 cc. of a milky suspension of R variant, and a similar group infected with the same dose of S. An animal from each group was killed at 24 hours, 48 hours, and at 7 days. The injection was incomplete in the second S animal. The peritoneal fluid, omentum, and peritoneal lymph nodes were examined supravitally, and sections were taken of the abdominal wall at the site of injection, the omentum, and the lymph nodes.

The R reaction was slow and of mild intensity. The enlargement of the "milk spots" was moderate and discrete. Necrosis of tissue was slight and up to 7 days

occurred only at the site of injection. A moderate increase of neutrophiles occurred during the first 2 days but decreased later. Phagocytosis by clasmatocytes occurred during the 2nd day, but decreased later. The number of fibroblasts and monocytes was slight at 1 day, but it increased progressively in the next two specimens. The monocytes had often matured to epithelioid cells in the 7 day animal. Tubercle bacilli were present in small and decreasing numbers in the peritoneal fluid but there were none to be found in the tissues. Acid-fast granules were seen in the phagocytes of the lymph nodes.

The S reaction was rapid and more intense. The "milk spots" were larger at 1 day, they increased in size progressively later, and the interspaces were partially obliterated. Necrosis (abscesses?) in the omentum were seen at 1 day and later. Large numbers of bacilli were found in the peritoneal fluid, omentum, and lymph nodes. Neutrophiles, often fragmented, were found at 1 day but they had disappeared at 7 days. Phagocytosis was marked in the first animal and absent later. The fibroblastic reaction was slight. Mononuclear cells developed in large numbers early and were present in all stages of stimulation, including many epithelioid cells at 7 days.

In the present study the supravital method confirmed the knowledge obtained by fixed tissue study. It noted in addition the presence of clasmatocytes at early dates. The sections permitted recognition of all other elements, as well as the structural relationships.

Summary of Pathology.—There is a marked difference in virulence, as demonstrated by pathogenicity, between typical H<sub>37</sub> R and S variants. The R variant has a low virulence and is very slightly pathogenic. It caused no mortality per se, very little gross evidence of tuberculosis, and a correspondingly small number of microscopic lesions. Animals killed after 4 to 6 weeks contained few lesions or none. Tubercle bacilli were occasionally present in small numbers at early dates, which decreased and were absent later. Microscopically, the lesions were small, usually unprogressive, were composed chiefly of proliferation, very rarely contained caseation, and regressed early (the so called "hard" tubercle).

The S variant had a high virulence and was highly pathogenic. It produced large, widespread lesions in the gross, with a correspondingly large microscopic involvement, and often resulted fatally. The lesions were usually progressive, were composed of proliferation with early and considerable abscess formation and caseation (the "soft" tubercle), and there were often attendant allergic phenomena, contiguous necrosis, and anemic infarction. These latter conditions were most

notable at a certain period of the infection (4 to 6 weeks). Tubercle bacilli were found in large numbers, especially during the period mentioned, but decreased in surviving animals.

The generalities mentioned above were seen to be modified by several factors: species, route of infection, dose, variation of dissociation, duration of infection, and secondary infection.

Rabbits, considering the dosage given, were less susceptible to both the R and S variants than guinea pigs, as they are to undissociated cultures. Their lesions were less progressive in appearance, abscess formation was rare, regression was early, and giant cells were more common.

The portal of infection further modified the disease. The intratesticular route in guinea pigs allowed a rapid spread and considerable disease production. The subcutaneous route not only decreased the rapidity of spread and the amount and type of disease (better controlled, less allergic) but, possibly because of the irregular delays of bacillary ingress, produced some irregularity in development. The intravenous route in rabbits allowed considerable disease and a good differentiation between variants. Intratesticular infection in this species also allowed a regular development of disease by the virulent strain, but the subcutaneous portal again held up both types of variant too completely for contrast.

The infecting dose is important in forming a comparative picture. Less than 100,000 bacilli should be sufficient by testicle in guinea pigs; at least 100,000 is necessary subcutaneously. A milligram is required to infect rabbits by any route. The R variant cannot supervene in any of these quantities; the S variant does so as completely as it is able. Should the virulence of these variants be more completely refined in the future, larger and smaller doses would be necessary to test virulence. The single best method at present would be inoculation of 10,000 to 100,000 bacilli intratesticularly in guinea pigs.

Variants of different dissociates, or from replants of the same dissociation, have slightly different levels of virulence. It would seem that the infections of Series 4 (500,000 bacilli) were both slightly less severe than those of Series 2 (100,000 bacilli). The infection obtained from the cultures used in Series 10 seemed more severe than those in Series 5 (1 mg. intravenously in rabbits). The results of inoculation

of the atypical, intermediate colonies used in Series 1 (5,000,000 intratesticularly in guinea pigs), producing lesions with relatively slight differentiation, have been mentioned.

There were definite changes in pathology dependent on the duration of infection. The R variant often caused small lesions at early dates, especially in guinea pigs, which regressed completely later. The disease caused by the S variant reached its height at from 4 to 6 weeks. There was evidence of a tendency to chronicity in the animals surviving this period, as evidenced by less rapid extension, resolution, decrease in the number of bacilli, and a remission from signs of allergy.

Secondary infections possibly had an effect on the tuberculous process. Rabbits with S infection and snuffles died during the height of the tuberculosis. The snuffles became evident or was intensified during this period (2 to 6 weeks) in both R and S inoculants.

It is clear that the factors which are discussed were interdependent. The relative age, weight, and the sex of the animals had no demonstrable effect on the progress of the disease. The first two factors were quite constant, the third was partially dictated by the routes of infection.

#### DISCUSSION

In the preceding pages we have presented the successful dissociation of a human type of tubercle bacillus,  $H_{37}$ . From the original culture two extreme types of variants have been isolated and a description of their physical and biological characteristics has been given in the text.

To obtain a successful dissociation by the method described the following procedure must be carefully observed: (1) Cultivation of the culture on glycerol potato bile medium for at least 4 months. (2) Subculture on special media with a suspension so dilute that the resulting growth will be in single colonies. (3) Resuspension of picked typical colonies in a synthetic fluid medium and seeding of its filtrate in new flasks. (4) Assortment in this manner through at least six platings. Patience is the greatest asset in studies such as this, with the development of the ability to recognize typical colony morphology.

Virulence, defined as the relative ability of the particular strain in question to grow in normal individuals of an animal species which is naturally susceptible to the type from which the strain is derived,

is therefore the growth factor. It is the *in vivo* manifestation of propagation and it depends on some as yet undefined constituent. Colony morphology is the *in vitro* manifestation of growth and it too depends on the bacillary constituents. We have shown that the virulence of the H<sub>37</sub> strain is definitely associated with the colony morphology. The growth factor of the bacillus is therefore the link between the culture and the lesion.

The result of dissociation of a strain forces a reconsideration of the host-parasite relationship in which it may be involved. Its virulent variant may grow in a species which is not naturally favorable soil; the avirulent variant may fail to propagate in an ordinarily susceptible species. The responsibility for virulence thus belongs increasingly to the bacillus and less to the host.

The factors which are known to modify the development of tuberculous infection have been mentioned before (11, 12). The present studies have reaffirmed their importance and interrelationship. The effect of virulence as the most important of these is emphasized. In order that species of animal, route of infection, and dosage of bacilli be the only important modifying factors of pathogenesis by the two variants, the bacilli for all inoculations should have been derived at once from a single pair of cultures. The present method, however, has allowed a contrast between the virulence of subcultures and an assay of the value of colony morphology in predicting pathogenicity.

We are still engaged in further experiments on the problem of the cellular reaction to the variants, and its relationship to immunity and hypersensitivity. Preliminary results would indicate that each variant causes a pathogenesis which is a result of its being (1) particulate matter, and (2) of a certain composition (?), and (3) of a certain virulence. The first mentioned character dictates the immediate cellular reaction, which is considered to be of the same type for various bacilli and other irritants (8, 12, 13, 14, 15). The present experiments demonstrated that the secondary cellular reaction (24 and 48 hours) was greater for the virulent variant; that destruction of the less virulent variant began very soon; and that these changes occurred before a general sensitization could account for the difference.

The relative spread of bacilli and the maturity of the tubercle depend on virulence (growth) with the resultant differences in number of lesions, maturity of mononuclears (phosphatid), and tendency to necrosis (protein sensitization). Since the maturity and necrosis varied directly with the number of bacilli present, proof of the effect of the chemical composition factor is lacking in pathogenesis. That a difference in composition is present is suggested by the results of sensitization with heat-killed variant cultures.

Healing appeared to be non-specific and its type dependent upon the stage of tubercle from which it developed.

It is quite possible that there will be a further change in virulence of the two variants following repeated cultivation. The present series of subcultures demonstrated an increasing differentiation. Other strains have been refined, chiefly in the direction of avirulence, e.g. by long cultivation (B.C.G.,  $R_1$ ). The decrease in virulence of the  $H_{37}$  R, when compared with the parent culture, is more notable than the increase in virulence of the S. This is probably due to the original relatively high virulence of the  $H_{37}$  strain.

It is also possible that other means of forcing a dissociation may be discovered.

We believe it is becoming clear that in many important problems in the bacteriology and pathology of experimental tuberculosis one must take into consideration the phenomenon of bacterial instability, and especially that aspect which has been termed bacterial dissociation. The subject is still in an unsettled state, but the studies reported here give us hope that perhaps our efforts are in the right direction.

#### CONCLUSIONS

- 1. The H<sub>37</sub> strain of human tubercle bacillus has been dissociated into two variants,—the R, or avirulent, and the S, or virulent, variants.
- 2. When their dissociation is complete, colony morphology for each is distinctive and typical.
- 3. The morphology of the individual bacilli of the two variants is different and characteristic.
- 4. The R variant develops best in an acid medium; the S develops best in an alkaline medium.
- 5. A method of assortment to facilitate the procedure of forced dissociation has been described.
- 6. The difference in virulence has been proven by observations on the pathogenesis of the variants.

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### EXPLANATION OF PLATES

### PLATE 31

- Fig. 1. R variant; gentian violet egg medium, pH 6.1; 60 day growth. Typical, discrete, crater-like or "worm-cast" colonies, dry, upright, slightly chromogenic, and with relatively clear-cut peripheries. ×2.
  - Fig. 2. R variant; same plate as Fig. 1.  $\times 5$ .
- Fig. 3. S variant; gentian violet egg medium, pH 7.2; 60 day growth. Typical, wrinkled or stippled, cream-colored colonies, only slightly raised, and with spreading veil-like peripheries. Normal size.
  - Fig. 4. S variant; same plate as Fig. 3. ×5.
- Fig. 5. S variant; fixed smear from 35 day culture, gentian violet egg medium, pH 7.2; Ziehl-Neelsen stain. Short rods, occasionally curved, and with rare beaded forms. ×865.
- Fig. 6. R variant; fixed smear from 35 day culture on gentian violet cgg medium, pH 6.1, Ziehl-Neelsen stain. Pleomorphic acid-fast and non-acid-fast bacilli, usually larger than the S type. Club-shaped, filamentous beaded, and granular forms are present. ×865.
- Fig. 7. Intermediate colonies; gentian violet egg medium, pH 6.1; 60 day growth. (a) R? colonies, twisted, slightly moist coils with slight chromogenicity, intermingled with (b) S? colonies, with spreading peripheral veils, a cream color, and slightly raised and stippled centers.

- Fig. 8. S variant; Proskauer and Beck synthetic medium, pH 7.2; 30 day growth. A fine, spreading, slimy, veil-like, surface growth with few dense areas. ×2.
- Fig. 9. R variant; Proskauer and Beck synthetic medium, pH 6.4; 30 day growth. A substantial growth in discrete, fenestrated, raised islands. ×2.

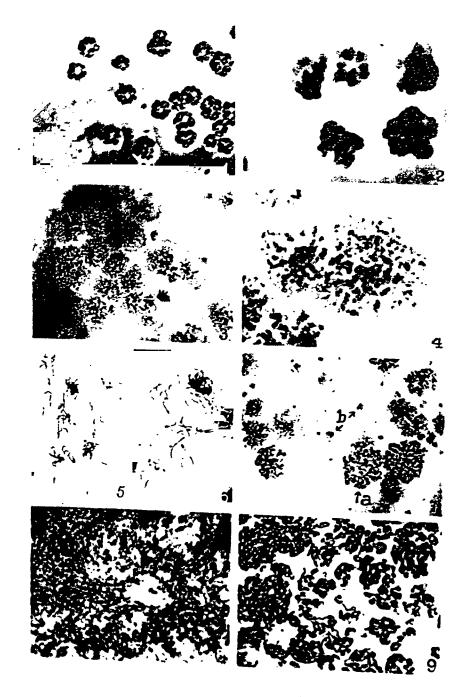
#### PLATE 32

- Fig. 10. R infection, 100,000 bacilli intratesticularly. Lung of a guinea pig killed at 63 days. An interstitial alveolar lesion formed near a small vessel and composed of slightly developed epithelioid cells, monocytes, and lymphocytes. No bacilli could be found. ×600.
- Fig. 11. R infection, 100,000 bacilli intratesticularly. Liver of a guinea pig killed at 98 days. A portion of a well localized, sluggish tubercle originating in a portal space. Same cellular composition as Fig. 10. There were no bacilli visible. ×400.
- Fig. 12. S infection, 100,000 bacilli intratesticularly. Lung of a guinea pig dead of tuberculosis at 36 days. A "soft" acute interstitial tubercle with abscess composed of necrotic epithelioid cells and neutrophiles. Many tubercle bacilli were seen in epithelioid cells and debris. ×400.
- Fig. 13. R infection, 1 mg. of bacilli intravenously. Liver of a rabbit killed at 27 days. The lesion consists almost entirely of Langhans giant cells situated intersinusoidally. Bacilli were not seen. ×600.
- Fig. 14. S infection, same animal in Fig. 12. An exudative pneumonia in cluster-like distribution. It is composed of whole and fragmented neutrophiles and epithelioid cells. Many bacilli were found in cells and debris. ×400.

### PLATE 33

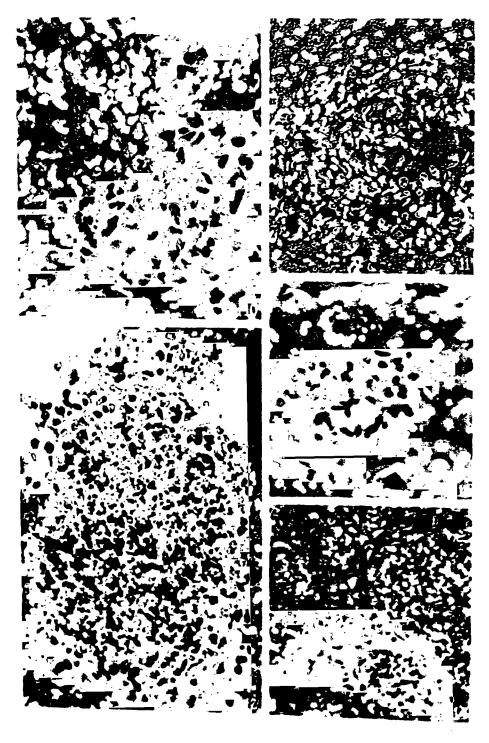
- Fig. 15. S infection, 100,000 bacilli intratesticularly. Lung of a guinea pig dead of tuberculosis at 36 days. Small abscesses with acute caseation in tissue contiguous to and in the walls of a large vessel. There were many bacilli present. ×55.
- Fig. 16. S infection, 100,000 bacilli intratesticularly. Liver of a guinea pig dead of tuberculosis at 41 days. An acute lesion, with abscess and early cascation, in a portal space. The isolated bile ducts are seen peripherally, and parietal to those are occasional large areas of necrotic liver cells (not shown). The lesion contains many bacilli. ×55.
- Fig. 17. S infection, 1 mg. of bacilli intravenously. Lung of a rabbit killed at 32 days. Cascation of tubercle (at bottom) can be seen, with a proliferative pneumonia above. The predominant cell is the epithelioid. Many bacilli were present. ×600.
- Fig. 18. S infection, 5,000,000 bacilli intratesticularly. Spleen of a guinea pig dead of tuberculosis at 64 days. A central island of cascation is surrounded by fibrin strands and an exudate of serum and erythrocytes. There is a complete loss of organic structure due to the allergic swelling. Great numbers of poorly staining bacilli were seen in the exudate and cascation. ×55.

Fig. 19. S infection, 500,000 bacilli intratesticularly. Liver of a guinea pig dead of tuberculosis at 97 days. A portion of a huge proliferative lesion characteristic of the older disease. It is sluggishly progressive, quite discrete, contains a small amount of caseation (left) and many lymphocytes. A single bacillus was found in this lesion.  $\times 55$ .

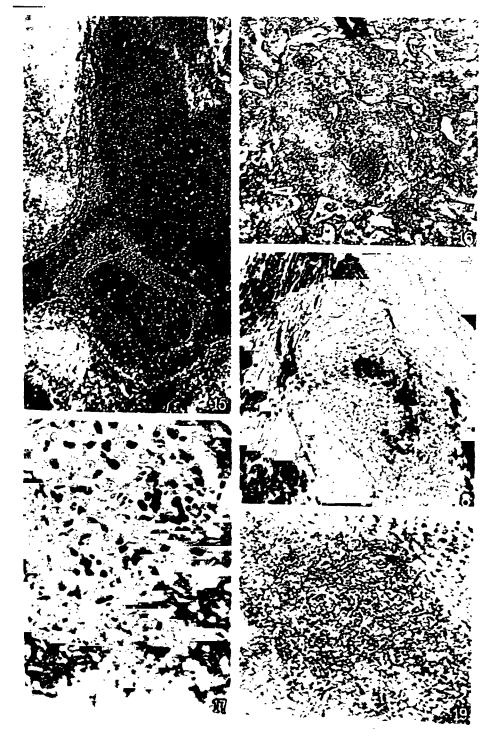


. Strenker at all . To be the  $k_{\rm B} T \gtrsim 111$ 











# THE PROTECTIVE ACTION OF COPPER AGAINST TRYP-ANOSOMA EQUIPERDUM INFECTION IN ALBINO RATS\*

By DAVID PERLA, M.D.

(From the Laboratory Division, Montefiore Hospital, New York)

(Received for publication, July 17, 1934)

In previous work it was found that the addition of small amounts of copper to an adequate diet protected a large percentage of rats from Bartonella muris anemia following splenectomy (1). In subsequent experiments the effect of supplements of copper to the diet on the natural resistance of adult rats to Trypanosoma lewisi infection was studied. The addition to an adequate diet of copper in amounts equivalent to 0.1 mg. of elemental copper per rat per day or of iron in amounts equivalent to 1 mg. of elemental iron per day, or both, during a period of 10 days prior to an induced infection with Trypanosoma lewisi raised the natural resistance of the rat to the disease. The infection was completely aborted in almost 50 per cent of instances (2). Supplements of lead salts in equivalent amounts, used as control, had no effect.

In the present communication the effect of additions of copper salts to the diet on a subsequently induced infection with Trypanosoma equiperdum was determined. In preliminary experiments the infection was carefully standardized for our stock of rats and the pathology of the disease studied (3). The virulence of this trypanosome for the white rat is so great that it was essential first to establish the critical dose of organisms that results in a fatal infection and to determine the duration of the disease with varying numbers of parasites.

Following the standardization of the infection in normal rats the effects of copper supplements on the course of the infection was

<sup>\*</sup> Read before the Federation of American Societies for Experimental Biology, New York, March 31, 1934.

studied in 4 groups of rats infected with varying quantities of trypanosomes.

The rats used in these experiments have been raised in our laboratory for many years and maintained under constant environmental and dietary conditions. The diet consisted of 15 gm. per rat per day of a mixture composed of hominy 100 parts, rolled oats 25 parts, fine meat and bone scraps 25 parts, dried skimmed milk 16 parts, and salt 1½ parts. Twice a week the rats received whole milk and bread ad lib. and greens (lettuce leaves). The exact copper content of this diet was difficult to estimate, but the food mixture was found to contain about 0.025 mg. of elemental copper per 15 gm. of food. In the experiments reported the copper was added in the form of copper sulfate in amounts equivalent to 0.2 mg. of copper per rat per day. Lactose was used as a vehicle. The iron was added in the form of iron ammonium citrate in doses equivalent to 2 mg. of elemental iron per day.<sup>1</sup>

The experiments were divided into 4 groups. In the first group, 20 million trypanosomes were used as the infecting dose; in the second group, 100,000 trypanosomes; in the third group, 10,000 trypanosomes; and in the fourth group 2,000 trypanosomes. In the first 3 groups the effect of additions of copper to an adequate diet on the course of the subsequently induced infection was studied. In the fourth group the effect of additions of copper or iron to the diet on the subsequently induced infection was determined.

The Effect of Copper Supplements to an Adequate Diet, on Trypanosoma equiperdum Infection Induced with Large Numbers of Parasites

The rats were divided into 2 groups. Group I consisted of 30 rats. Of these, 10 received daily supplements of copper as copper sulfate in amounts equivalent to 0.2 mg. of elemental copper per day, and 20 rats were fed on the normal diet. The supplements were commenced 10 days prior to the injection of blood of trypanosome-infected rats. Each was injected intraperitoneally with 20 million trypanosomes. Group II consisted of 16 rats. Of these, 8 received copper supplements as in Group I, during a period of 10 days prior to injection of trypanosomes, and 8 received the normal diet. Each of these rats was injected intraperitoneally with 100,000 trypanosomes.

Results.—All the rats of these 2 groups succumbed to the infection. There was no essential difference in the course of the disease, but a

<sup>1</sup> I am indebted to the Myron L. Walker Co., Inc., of Mt. Vernon, New York, for the copper and iron preparations.

slight prolongation of the infection in the copper-fed group was noted (see Table I). No effect on the pathology of the disease was observed.

# The Effect of Copper and Iron Supplements to an Adequate Diet on Trypanosoma equiperdum Infection Induced with Small Numbers of Parasites

In these experiments 65 rats were used. These were divided into 2 groups. In the first, 8 rats received copper supplements to the diet as in previous experi-

The Effect on Trypanosoma equiperdum Infection in Adult Albino Rats of Copper and Iron Supplements to an Adequate Diet\*

The Effect on I	Trypanosoma eq	uiperdum Inje Supplements to	an Adequate	Diel*	Average
		No. of trypano-	No. of rats with abortive infection	Per cent of rats with abortive infection	duration of life in fatal infections
No. of rats	Supplement (i	intraperitoneally)			days 4.5
	Coppert	20 million 20 million	0	0	3.6
10 20	Controls	100,000	0	0 0	7.5
8 8	Copper Controls	100,000	6	75	9.6
S	Copper Controls	10,000	1	0	1
8 20	Copper	2,000	0 1 10	100 100 52	14.5
10 19	Iron‡ Controls	2,00	ced 10 days	prior to the	injection of the
	-loments	were commen	-		0.2 mg 0

<sup>\*</sup> The supplements were commenced 10 days prior to the injection of the

ments and S were fed on the normal diet. Each of these was injected intraperitoneally with 10,000 trypanosomes. In the second group, 20 rats received copper supplements as above, 10 received supplements of iron in amounts equivalent to 2 mg, of elemental iron per rat per day during the same period and 19 were fed on the normal diet. Each of these rats was injected with 2,000 trypanosomes intraperitoneally.

<sup>†</sup> The copper was given as copper sulfate in amounts equivalent to 0.2 mg. of trypanosomes.

<sup>†</sup> The iron was given as iron ammonium citrate in amounts equivalent to 2 mg. elemental copper per rat per day. of elemental iron per rat per day.

Results.—With 10,000 trypanosomes as the infecting dose all the controls succumbed to the infection. The average duration of life was 9.2 days. Of the copper-fed group, 75 per cent developed no obvious evidence of infection (abortive infection) and survived. No parasites were observed in smears of the blood. With 2,000 trypanosomes as the infecting dose, 48 per cent of the controls developed severe infections and died, but all of the rats which had received copper and iron supplements to the diet had abortive infections and survived.

From these experiments it may be observed that additions of copper or iron to an adequate diet in the rat definitely raise the natural resistance of this animal to a subsequently induced infection with *Trypanosoma equiperdum*.

#### DISCUSSION

The importance of copper in the resistance of albino rats to three types of infection, with Bartonella muris, Trypanosoma lewisi, and Trypanosoma equiperdum respectively, has been indicated by the experiments reported in previous studies and in the present one. The rat is an ideal animal in which to study the effect of copper on resistance, as the diet of the rats used in most laboratories contains a minimal amount of copper (sufficient, however, to prevent nutritional anemia).

Cunningham in a study of the relative amounts of copper in various animal and plant tissues found that the rat has less copper in its organs than any other animal studied (4). The liver of the rat contains 1/20 to 1/30 the percentage weight of copper found in the liver of the rabbit or the guinea pig. Individual variations in the resistance to Bartonella muris, Trypanosoma lewisi, and Trypanosoma equiperdum, may be dependent on the copper content of the food of these animals. It is of interest that the guinea pig, an animal low in resistance to many infections, is refractory to Trypanosoma lewisi and Bartonella muris and develops a chronic infection with Trypanosoma equiperdum. The copper content of its diet and tissues is high. The rat, highly resistant to most infections, is very susceptible to these three diseases. The copper content of its diet and tissues is low. The results of the experiments reported do not suggest, however, that

an excess of copper in a diet already adequately supplied with this element will increase the resistance of the individual above the normal level.

It is of significance that the species susceptibility of the rat to certain infections may be markedly altered by adding copper or iron to the diet prior to infection. It is probable that the protective action is dependent on the fact that copper is a catalytic oxidative agent in cellular metabolism. In rats the copper content of the tissues, while sufficient to prevent a nutritional anemia, may result in a depression of the threshold resistance of the cell to certain types of injury.

These studies on the rôle of copper in the diet in natural resistance to infections emphasize again the importance of maintaining the conditions of diet, environment, and strain of animals constant in experimental studies on infection and resistance. No doubt variations in resistance observed in apparently identical infections in different laboratories are due to subtle variations in strain, diet, and environment.

#### SUMMARY

The effect was studied of additions of copper to an adequate diet on the course of infection with Trypanosoma equiperdum in rats. Copper in amounts equivalent to 0.2 mg. of elemental copper per rat per day during a period of 10 days prior to an induced infection with small numbers of trypanosomes raised the natural resistance of the rat to the infection. The infection was aborted in all instances when the rats were infected by the injection of 2,000 trypanosomes and in 75 per cent of instances when the rats were infected by the injection of 10,000 trypanosomes.

#### CONCLUSION

The natural resistance of the rat to infection with *Trypanosoma* equiperdum can be markedly raised by supplements of copper to the diet prior to infection.

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### STUDIES ON MOUSE LEUKEMIA

## XI. METABOLIC EFFECTS OF HOST CONSTITUTION

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The preceding study in this series, on the metabolism of cells of transmissible lymphatic leukemia (9), showed greater consistency in repeated observations than is reported by others for neoplastic tissue (1, 2, 5, 10). Since no other account has included a statement as to genetic uniformity of hosts, it seemed possible that the high variability of other results on tissue metabolism might be related to variability of hosts. The experiments herein recorded test directly the effects of genetically different host constitutions upon the metabolism of given lines of leukemic cells.

#### Material

The highly inbred strains of mice reported in earlier papers of this series by MacDowell and Richter (3, 4, 6) and two of the transmission lines of cells of lymphatic leukemia proved especially well suited for this study.

The experiments have been carried out with mice, 5 to 8 weeks old, from the 26th to 32nd generations of brother by sister matings of Strain C58 and from the 29th to 31st generations of brother by sister matings of Strain Storrs-Little. More than half of the mice from each strain came from the 30th generation. For observations on the normal metabolism of the lymph nodes, 48 mice from each strain were studied. Leukemic cells were used of Line I, Transfers 264-275, and of Line M-liver, Transfers 55-67. The metabolism of these cell lines growing in mice of Strain C58 has already been reported (8, 9).

The same doses of the same saline suspensions of cells were inoculated into mice of Strains Storrs-Little and C58. With cells of Line M-liver 100 per cent of 107 mice in Strain C58 died 3.5-6 days after inoculation, and 47 per cent of 59 mice in Strain Storrs-Little in 4-9 days. With Line I, 100 per cent of 52 mice died in 3.5-5 days in Strain C58, and 48 per cent of 60 mice in Strain Storrs-Little in 4-7 days.

Line M-liver was carried through Strain Storrs-Little for two and three transfers and then successfully transferred back to Strain C58 in 100 per cent of 16 mice. Line I, after one transfer through Storrs-Little mice was successfully returned to C58 hosts in 52 per cent of 25 mice.

### Method

The method of observing metabolic rates used in this study is identical with that of the previous experiments (9). The oxygen consumption and aerobic and anaerobic glycolysis were measured with the Fenn modification of the Thunberg differential volumeter. Infiltrated nodes were taken at the time after inoculation when the infiltration of leukemic cells had become so extensive that the normal architecture of the gland had been completely obliterated. To determine the time required for this change, special histological studies were made with independent material with each of the lines in both strains during the course of these experiments. Judged by the degree of infiltration and number of mitotic figures in the involved areas, the distribution of lesions and the rate of growth of the inoculated cells are the same in Strain Storrs-Little as in Strain C58 for the first 3-5 days. In Strain Storrs-Little at this period no evidence was found of the necrosis and resorption of leukemic cells that subsequently appears in many cases in this strain in connection with recovery. This point is confirmed by cytological observations of tissues actually used in these experiments. From study of 50 sets of tissue taken from respirometers, no difference could be detected in staining reactions, occurrence of pycnotic nuclei, and degenerating cells from tissue fixed at death; and leukemic tissues from Strains C58 and Storrs-Little do not differ. Thus strain differences in the amount of living infiltrations due to unexpectedly early retrogression in Storrs-Little hosts are not involved in the general results.

The cervical, abdominal, and inguinal lymph nodes were studied. To obtain sufficient material for determination of the normal oxygen consumption, aerobic and anaerobic glycolysis, lymph nodes of four mice of the same sex and almost invariably from the same litter were combined. Each leukemic mouse usually gave sufficient material for a determination, although it was occasionally necessary to combine tissues from two or three mice. The nodes were immersed in glucose-free Ringer's solution and cut into slices 0.2–0.3 mm. thick and then weighed on a torsion balance accurate to 0.2 mg. 30–100 mg. of tissue, moist weight, were placed in each respirometer. The moisture content was estimated as 80 per cent and corrected by this factor (7).

The oxygen consumption was measured directly with the tissue in Ringer's solution of 0.9 per cent NaCl, 0.022 per cent KCl, and 0.0236 per cent CaCl<sub>2</sub>, containing 0.2 per cent glucose. In some cases a phosphate buffer of pH 7.4 was used. The presence of the buffer did not influence the rate or duration of a constant oxygen use. CO<sub>2</sub> was absorbed by a 0.1 N NaOH solution placed in an inset. A control respirometer contained tissue immersed in glucose-free Ringer's solution to which 0.025 M NaHCO<sub>3</sub> was added. The solution was equilibrated with a gas mixture

of 5 per cent  $CO_2$  and 95 per cent  $O_2$  by bubbling the mixture through it for  $\frac{1}{2}$  hour. Then the gas mixture was run through the experimental bottle, after the tissue had been placed in the Ringer bicarbonate solution, for another 10 minutes. Flushing of the bottles with the gases was insured by having an outlet through a stop-cock attached to them. The excess aerobic and anaerobic glycolysis was measured with 0.2 per cent glucose in the Ringer-bicarbonate solution and by equilibrating the tissue and solution with 5 per cent  $CO_2$  and 95 per cent  $O_2$  for the aerobic, and 5 per cent  $CO_2$  and 95 per cent  $O_2$  for the aerobic determinations. The pH in this bicarbonate system, according to the Henderson-Hasselbalch equation, is 7.44. The respirometers were shaken 140–160 times a minute through an excursion of 5 cm. The temperature was 37.5°C.  $\pm 0.005$ °C. At the conclusion of a metabolic determination the tissue in the respirometer was fixed in Flemming's fixative for microscopical study.

The results are expressed as follows:

$$Q_{\text{O}_1} = \frac{\text{c.mm. oxygen consumed}}{\text{mg. dry weight per hour}}$$

$$Q_{\text{CO}_1}^{\text{O}_1} = \frac{\text{c.mm. excess aerobic glycolysis}}{\text{mg. dry weight per hour}}$$

$$Q_{\text{CO}_1}^{\text{N}_2} = \frac{\text{c.mm. excess anaerobic glycolysis}}{\text{mg. dry weight per hour}}$$

#### RESULTS

In Table I are the individual determinations of the rates of oxygen consumption and aerobic and anaerobic glycolysis of the normal lymph nodes of Strains C58 and Storrs-Little. In Table II are the dates, transfer number, interval after inoculation, and individual determinations of the metabolism of lymph nodes of mice of Strains C58 and Storrs-Little inoculated with cells of Line M-liver. A of this table are the observations for the line in Strain C58; in Part B the observations in Storrs-Little (the second column giving the number of transfers in Storrs-Little); and in Part C the metabolism of the cells on return to Strain C58 from Storrs-Little. Table III is similar to Table II; the results are concerned with Line I instead of M-liver. In Table IV, the first part is a summary of the means, their probable errors, and the standard deviations for these different lines of cells and strains of mice. The second part is a statistical analysis of the differences observed. Differences that are 3 or more times their probable errors are considered statistically significant. These have been set in bold faced type. Fig. 1 represents the arithmetic means and their probable errors of the metabolic rates of leukemic cells of Lines I and M-liver, in Strain C58 and in Strain Storrs-Little.

TABLE I

Rates of Oxygen Consumption and Aerobic and Anaerobic Glycolysis of Lymph

Nodes of Uninoculated Mice of Strains C58 and Storrs-Little—

6-8 Weeks Old

Q <sub>O2</sub>	Q <sub>CO2</sub>	G <sub>V</sub> <sup>CO</sup>							
Strain	n C58 uninoculated—48 mice	used							
5.0	2.3	4.4							
7.0	3.0	3.2							
4.7	2.0	3.4							
6.5	2.0	4.7							
5.5	1.9	4.4							
4.4	2.1	4.4							
5.4	2.6	6.3							
5.1	1,1	7.2							
5.4	1.1	8.1							
5.1	2.3	8.0							
5.9	2.4	6.8							
5.4	2.7	8.4							
Strain Storrs-Little uninoculated—48 mice used									
5.8	1.5	5.5							
6.0	2.4	3.9							
6.6	1.1	5.7							
5.5	3.1	6.3							
6.5	2.4	6.4							
5.5	3.0	6.3							
5.0	2.0	7.1							
5.4	1.4	6.9							
5.6	1.5	5.4							
5.6	3.6	8.5							
5.1	2.8	8.9							
4.9	2.8	7.1							

These data and summaries reveal the following:

The Genetic Constitution of the Host Affects the Metabolism of Transmitted Leukemic Cells.—The five differences in bold faced type in the

TABLE II

Line M-Liver in Lymph Nodes of Hosts from Strains C58 and Storrs-Little

	Length of		Metabolic r	ates of lym	ph rodes			
No. of successive transfers of Line M-liver a hosts of each strain at time of observation	time after inoculation	Date	Q <sub>O2</sub>	Q <sub>CO2</sub>	6 <sub>Z</sub> ;			
Part A. Hosts f	rom Strain	C58-24 mice	used					
	days	1933	1					
C58 55	4	Jan. 19	7.5	7.9	19.4			
" 55	4	" 19	4.9	7.2	18.4			
" 56	4	" 23	6.9	6.8	23.7			
" 56	4	" 23	6.0	9.8	19.3			
" 57	4	" 27	5.2	8.8	21.2			
" 57	4	" 27	5.7	9.9	19.1			
" 57	4	" 27	5.5	7.7	17.5			
<b>"</b> 58	4	" 30	5.4	9.4	20.1			
<b>"</b> 58	4	" 30	5.6	10.6	21.5			
<b>"</b> 58	4	" 30	6.7	6.0	16.1			
<b>"</b> 63	4	Feb. 20	4.7	8.1	20.3			
" 63	4	" 20	5.3	8.6	20.7			
Part B. Hosts from Strain Storrs-Little—20 mice used								
C58 61 Storrs-Little 1	5	Feb. 12	6.1	4.0	11.0			
" 61 " 2	5	" 16	6.4	4.7	13.5			
" 61 " 1	5	" 18	6.3	5.2	16.1			
" 61 " 1	5	" 18	6.1	5.4	14.9			
" 61 " 3	5	" 22	5.4	4.5	10.1			
"66 "2	5	Mar. 13	6.2	4.5	11.9			
" 67 <b>"</b> 2	6	" 15	6.2	5.0	13.4			
" 67 " 2	6	" 15	6.6	4.8	14.7			
" 67   "     3	7	" 22	5.0	4.1	11.7			
"66 " 4	6	" 25	5.4	4.4	14.8			
Part C. Hosts from Strain C58	following t	ransfers in St	orrs-Little	10 mic	e used			
C58 61 Storrs-Little 3 C58 1	6	Feb. 28	5.6	7.2	20.8			
"66 "2"1	3	Mar. 16	5.5	7.8	19.5			
" 67 " 2 " 1	5	" 20	5.6	10.1	20.7			
" 66   "   3   "   1	4	" 23	5.0	7.8	20.0			
" 67 " 2 " 1	5	" 27	5.2	10.9	20.7			
				· · · · · · · · · · · · · · · · · · ·				

second part of Table IV provide the experimental evidence supporting this proposition. Each of these differences is in the metabolism of a line of cells in hosts of different constitution.

TABLE III

Line I in Lymph Nodes of Hosts from Strains C58 and Storrs-Little

No. of successive transfers of Line I in hosts	Length of	Metabolic rates of lymph nodes							
of each strain at time of observation	time after inoculation	Date	Q <sub>O2</sub>	Q <sub>CO</sub> 2	Q <sub>CO</sub> ,				
Part A. Hosts f	rom Strain	C58—20 mice	used						
	days .	1933		1					
C58 264	3	Feb. 23	5.9	7.0	16.2				
" 26 <del>4</del>	3	" 23	5.8	10.5	18.8				
" 266	4	Mar. 3	4.9	8.4	20.2				
<b>" 268</b>	4	" 10	5.9	7.6	20.4				
<b>"</b> 272	4	" 24	5.0	8.4	16.0				
<b>"</b> 272	4	" 24	5.6	7.4	15.0				
<b>"</b> 273	3	" 27	5.4	8.6	19.0				
<b>"</b> 273	4	" 28	5.1	6.2	18.2				
<b>"</b> 275	3	Apr. 3	5.9	6.3	17.7				
<b>"</b> 275	3	<b>"</b> 3	5.1	7.4	18.7				
Part B. Hosts from Strain Storrs-Little—20 mice used									
C58 260 Storrs-Little 1	4	Feb. 10	6.2	2.7	7.5				
" 260 " 1	4	" 10	6.1	1.9	10.5				
" 260 " 1	5	" 11	7.1	2.5	7.9				
" 264 <b>"</b> 1	5	" 24	6.3	3.0	9.7				
" 264 " 1	5	" 24	6.6	3.2	11.2				
" 264 " 1	4	" 25	6.4	5.0	11.3				
" 269 " 2	5	Mar. 22	6.7	5.8	15.2				
" 269 <b>"</b> 2	6	" 23	6.8	7.3	13.7				
" 272 " 1	4	" 28	6.3	4.8	13.6				
" 272 " 2	4	Apr. 1	6.6	8.2					
Part C. Hosts from Strain C58 fo	llowing tra	nsfers in Stor	rs-Little-	-12 mice	used				
C58 269 Storrs-Little 1 C58 1	4	Mar. 21	5.7	7.6	19.8				
" 269 " 1 " 1	4	" 21	5.1	7.3	17.3				
" 272 " 1 " 1	3	" 31		7.5	19.6				
" 272 ". 1 " 1	4	Apr. 1	5.5	7.3	25.2				
" 273 " 1 " 1	4	" 5	6.0	6.5	19.0				
" 273 " 1 " 1	5	" 6	5.7	6.0	19.6				

However different the constitution of the hosts from these two strains may be, the metabolism of their lymph nodes before inoculation is not significantly different; as shown by this same table, the

TABLE IV

	S st Tables I to III: Means, Standard Deviations, and Differences	rabid Acans, Sta	Standard D	eviations, and 1	Differences		
	Store to Amunds	000		ι <sub>0</sub> ο		QCO,	
Line of Imberial cells	Strain of hosts	Mean	Standard	Mean	Standard	Mean	Standard deviation
Uninoculated	C58 (1) Storre-Little (2)	5.45±0.14 5.63±0.09	0.70	2.13±0.10 2.30±0.15	0.52	5.78±0.35 6.50±0.25	1.80
M-liver "	C58 Storrs-Little (4) C58 from Storrs-Little (5)	5.78±0.16 5.97±0.10 5.38±0.07	0.83 0.49 0.24	8.40±0.26 4.66±0.09 8.76±0.44	1.32 0.43 1.46	19.78±0.36 13.21±0.40 20.34±0.15	1.84 1.86 0.51
<b></b>	C58 (6) Storrs-Little (7) C58 from Storrs-Little (8)	5.46±0.08 6.51±0.06 5.60±0.09	0.39 0.29 0.30	7.78±0.26 4.44±0.43 7.03±0.17	1.20 2.04 0.62	18.02±0.36 11.18±0.56 20.08±0.68	1.70 2.47 2.46
Compation wil	Comparison with same line of cells continuously in hosts of Strain CS8	Difference	Difference P.R. difference	Difference	Difference P.E. difference	Difference	Difference P.E. difference
Uninoculated	Storrs-Little (2) minus (1)	+0.18:±0.17	1.06	+0.17±0.18	1.6.0	+0.72±0.43	1.67
M-liver 11	Storrs-Little (4) minus (3) CS8 from Storrs-Little (5) minus (3)	+0.19±0.19	1,00	-3.74±0.28 +0.36±0.50	13.36	- <b>6.67</b> ±0.54 +0.56±0.39	12.17
₩	Storrs-Little (7) minus (6) C58 from Storrs-Little (8) minus (6)	+1.05:±0.10 +0.14±0.12	10.50	-3.34±0.50 -0.75±0.31	6.68	-6.84±0.67 +2.06±0.77	10.21

greatest difference in means (anaerobic glycolysis) is less than twice its probable error, and the others are only about equal their probable errors.

In hosts of Strain Storrs-Little compared with Strain C58, Line I has a higher rate of oxygen consumption, but lower rates of aerobic and anaerobic glycolysis; Line M-liver has lower rates of aerobic and anaerobic glycolysis.

The statistical significance of these five differences is unquestionable; one is nearly 7 times its probable error and the others range from 10 to 13 times their respective probable errors. Among the ten other comparisons given in this table, the closest approach to statistical significance is 2.68 times the probable error of the difference. These results, however significant in themselves, can be interpreted only on the basis of the conditions of the experiment. Upon these depends the evidence that the differences observed are not concerned with changes in the cell lines themselves, and that the differences between hosts are genetic.

Changes in the characteristics of a line have been found according to all criteria, clinical, gross autopsy, interval before death, distribution of lesions, cytological traits, and metabolism. The metabolic rates of a line may be constant over long periods of successive transfers in the same hosts and then show a change. Several months before these experiments were started Line I and M-liver in hosts of Strain C58 gave different metabolic rates, whereas in these experiments the difference is shown to have disappeared. Accordingly, it was necessary to eliminate the possible influence of such a change by using the same transfer of the line of cells for both strains of mice, as indicated in Tables II and III. Since the results are not due to changes in the cell lines they must be due to differences between the host animals.

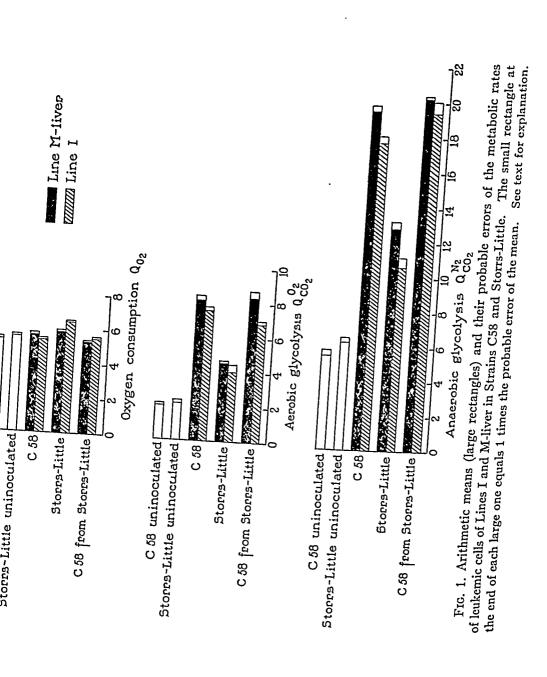
In regard to age, food, care, and experience in life, all the host mice were highly uniform. All were bred at Cold Spring Harbor and carried to New York by hand via train and subway soon after they were a month old. Since all were used between 60 and 80 days after birth, they spent roughly the same amount of time in the New York mouse room. Thus non-genetic differences between the two strains of hosts appear to have been eliminated. Positive evidence of genetic

differences is provided by the pedigree record of every mouse born in these strains. They differ in three genes for hair color, namely, black vs. brown, intense pigment vs. dilute, and pink eyes vs. dark eyes. Mice of Strain C58 are all black, as have been all those born in this strain since 1922; mice of Strain Storrs-Little are called pink-eyed, dilute brown, and all mice born in this strain since 1923 have been of the same description. These strains differ further in an undetermined number of genes influencing susceptibility of different lines of leukemic cells; one of these, necessary for the continued growth of Line I in one long period of its history, has been identified (4).

Non-genetic differences do not exist; genetic differences do exist. Whatever the nature of the differences between strains involved in the modification of the metabolism of the leukemic cells, it seems necessary to conclude that they are under the control of heredity. Under these conditions the effect of differences between these strains is open to investigation. The only point that can be indicated at present is that the metabolism of the normal lymphoid tissue is not concerned, since in this respect, as already pointed out, these strains are essentially alike.

The Effect of Host Constitution Varies with the Line of Leukemic Cells.—In Strain C58 the metabolism of Line I is similar in all three criteria to Line M-liver. Yet in passing to hosts of Strain Storrs-Little the metabolism of these lines is altered in different ways. The rate of oxygen consumption of Line I was significantly increased (difference is 10 times its probable error) in Strain Storrs-Little, while that of Line M-liver was significantly the same as in Strain C58 (difference equals its probable error). Although both lines in Storrs-Little show a similar change in means for aerobic and anaerobic glycolysis, a further difference in response of lines is indicated by the greater variability in determinations of aerobic glycolysis of Line I. The coefficients of variability of the aerobic glycolysis for Line I is 46 per cent, for Line M-liver, 10 per cent.

Thus the metabolic response to changes in their environment may reveal differences between lines that by all other criteria are indistinguishable. This extends to a considerable degree of subtility the previous conclusion that there are inherent differences between lines of transmissible lymphatic leukemia that modify their metabolism, and indicates the care necessary before concluding that two lines are alike.



JOSEPH VICTOR AND JAMES S. POTTER Host Constitution Does Not Modify Inherent Constitution of the Cell Line.—As soon as the cells are returned from Strain Storrs-Little to Strain C58, the metabolic rates return to their original level in C58 hosts. None of the differences between the metabolic rates before and after transfer through Storrs-Little hosts is statistically significant. In Line M-liver, after two and three transfers through Storrs-Little hosts, the differences for all three criteria have the opposite sign from the changes caused by the Storrs-Little hosts. In Line I, after one transfer through Storrs-Little hosts, the one difference that most closely approaches statistical significance (anaerobic glycolysis) varies in the opposite direction from the rate in Storrs-Little; in the other two criteria the deviation is in the same direction, but as noted,

These results indicate that the metabolism of these leukemic cells is modified by their immediate environment. Clearly enough a change in temperature will change metabolic rates; a change in hosts not significant. is on a higher level of complexity in its influence on metabolic rate. In both cases the changes are reversible. The metabolic rate observed for a given line is not a trait of the line as such; it is rather the reaction of a line of cells to a certain host. If the host can be defined the observation acquires significance, and can be repeated. In other hosts the reaction may be different, and yet the essential mechanism upon which the metabolic activity of the cell line depends may

These results both provide an interpretation of difficulties previously met in the field of metabolic studies on neoplastic tissue, and be constant throughout. establish the necessity for the use of host animals whose uniformity is controlled genetically and ontogenetically. To ignore temperature would be unthinkable; but the complicated specific differentia relationships of cell line and host strain, demonstrated herein, indicat that the most strict control of host animals from which neoplast tissue is taken can be considered of no less importance than t temperature under which the tissue is studied.

# SUNDIARY AND CONCLUSIONS

1. Two highly inbred strains of mice of different genetic cons tion (Storrs-Little and C5S) were used in a study of the influen hosts on metabolism of cells of transmissible lymphatic leuk The experiments were carried out with leukemic cells of transmission Line I as well as with Line M-liver. About 50 per cent of the Storrs-Little mice were killed by each line of cells at this time, while 100 per cent of the C58 mice were killed.

- 2. The normal lymphoid tissues of the two strains of mice were significantly the same in regard to rates of oxygen consumption and both aerobic and anaerobic glycolysis.
- 3. Leukemic cells of Line I, growing in hosts of Strain Storrs-Little, gave significantly lower rates of aerobic and anaerobic glycolysis than when growing in hosts of Strain C58. Oxygen consumption was significantly higher. Leukemic cells of Line M-liver, growing in hosts of Strain Storrs-Little, gave significantly lower rates of aerobic and anaerobic glycolysis than when growing in Strain C58. Oxygen consumption was not significantly different.
- 4. After one to three passages through hosts of Strain Storrs-Little, the cell lines were returned to hosts of Strain C58, with immediate return to significantly the same metabolic rates originally given by each line in hosts of Strain C58.
- 5. These results lead to the more general conclusions that: (a) The genetic constitution of the host modifies the metabolism of the cell line. (b) The same host constitution may modify the metabolism of different cell lines in different ways. (c) Host constitution does not appear to modify the inherent constitution of the leukemic cells, but acts as a determining environmental factor on their metabolism.

We are indebted to Miss Margaret R. Prest for assistance throughout this work.

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# THE ANTIBODY RESPONSE OF RABBITS TO INJECTIONS OF EMULSIONS AND EXTRACTS OF HOMOLOGOUS BRAIN

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Since the discovery of hemolysins by Bordet attempts have been made to demonstrate antibodies against practically every type of cell and tissue in the body. Most of the efforts were prompted by the idea that certain diseases, for example nephritis and diabetes, characterized by degenerative changes in the affected organs, might be due to the development of antibodies against the tissues of the particular organ involved. However, the early claims made for the specificity of organ antibodies have been discounted to such an extent by later more seasoned work that the theories evolved from them have been discarded in the majority of instances. In the period since 1926, Brandt, Guth, and Müller (1) and others (2, 3, 4) have demonstrated that brain tissue contains an alcohol-soluble lipoid which functions as a haptene and that when this lipoid is mixed with a heterologous protein a complete antigen is formed capable of inciting in animals the development of complement-fixing antibodies which are organ-rather than speciesspecific. This discovery again opens, for brain at least, the question of the etiological rôle of organ-specific antibodies in certain degenerative diseases and strengthens materially the hypothesis that the encephalomyelitis which follows antirabies vaccination, if not the entire group of postinfection encephalitides, is in some manner associated with the development of specific antibodies for brain.

#### EXPERIMENTAL

Since a number of workers (2, 4) have already shown that fresh emulsions of homologous brain possess little or no antigenicity and that only when a heterologous protein is added to the brain haptene does it become a complete antigen, we were interested to see whether certain degenerative processes such as autolysis are capable of altering

homologous brain material to such an extent that it becomes a complete antigenic complex. If this proved to be so, we planned to study the characteristics of the antigen as well as of the antibodies elicited by it. To this end rabbits were injected (1) with freshly prepared emulsions of rabbit brain, (2) with similar emulsions to which pig serum had been added, (3) with sterile emulsions of rabbit brain that had been allowed to stand at room temperature for 5 to 30 days, (4) with fresh emulsions prepared from rabbit brains experimentally infected with vaccine virus, and (5) with alcoholic extracts of rabbit brain plus pig serum. Following the injections sera of the animals were examined by means of complement fixation tests for the presence of antibodies against aqueous emulsions and alcoholic extracts of various organs.

### Methods

Rabbits.—Only young mature rabbits averaging 2000 gm. in weight were used. To insure a maximum uniformity in the antibody response, only pure-strain self-blue English rabbits received injections. The rabbits which received injections of emulsions of brain infected with vaccine virus were immunized 2 weeks prior to the initiation of the experiment by means of intradermal injections of 0.25 cc. of a 1:10 dilution of vaccine virus prepared in tissue culture according to the method of Rivers (5).

Emulsions of Fresh Brain.—5 gm. of brain tissue removed aseptically from a rabbit were ground in a mortar with alundum until thoroughly macerated. 80 cc. of Locke's solution were added by increments during the process of grinding until the tissue was well emulsified. The suspension was then centrifuged at low speed for 2 minutes and the supernatant fluid removed by means of a pipette. The inoculum was prepared by the addition of 10 cc. of Locke's solution to each 40 cc. of the supernatant fluid.

Emulsions of Fresh Brain Plus Pig Serum.—Emulsions of fresh brain plus pig serum were prepared in a manner similar to that just described for the preparation of emulsions of fresh brain, except that in the final step, 10 cc. of pig serum, instead of Locke's solution, were added to each 40 cc. of the centrifuged suspension.

Emulsions of Autolyzed Brain.—Brain tissue was ground directly to a 5 per cent emulsion in a manner similar to that described for the preparation of emulsions of fresh brain. The supernatant fluid from the centrifuged suspension was transferred to a sterile flask that was then closed by a cotton plug over which were placed several layers of tin-foil. This material was allowed to stand at room temperature during the course of the experiment. As required for the injections, portions of the emulsion were removed from 5 to 30 days after preparation. Before use and from time to time during the experiment samples of the emulsion were tested for the presence of bacterial contaminants by means of aerobic and anaerobic

cultures made in meat infusion broth (pH 7.8) and on blood agar. In no case during the course of an experiment were bacteria grown from emulsions found sterile at the start.

Emulsions of Brain Infected with Vaccine Virus.—A rabbit was inoculated intracerebrally with 0.25 cc. of a 5 per cent emulsion of rabbit brain infected with the Levaditi strain of vaccine virus. On the 3rd day after injection usually at the height of the infection the rabbit was killed. A 5 per cent emulsion in Locke's solution was prepared from the brain, the technique being similar to that described for the preparation of emulsions of fresh normal brain. The absence of ordinary bacterial contaminants in the brain was shown by means of aerobic and anaerobic cultures made in meat infusion broth (pH 7.8). On each occasion an additional rabbit was inoculated intracerebrally with a portion of the emulsion in order to furnish brain material for the next immunizing injection.

Alcoholic Extracts.—Alcoholic extracts of brain were prepared in the following manner: A known amount of tissue was thoroughly macerated in a mortar to which were added 5 cc. of 95 per cent ethyl alcohol for each gram of brain. The mixture was transferred to a flask which was then closed by a well fitting cork and allowed to stand at room temperature for 5 to 10 days. Then the material was passed through filter paper. In order to make immunizing emulsions 10 cc. of the filtrate were evaporated almost to dryness on the water bath and the residue was emulsified in 20 cc. of Locke's solution to which 5 cc. of pig serum had been added. For the experiments in which alcoholic extracts of organs other than brain were used the method of preparation of the extracts was the same.

Immunization of Rabbits.—Each rabbit received intraperitoneally 10 injections of 5 cc. of the brain emulsions or brain extracts. The inoculations were made at intervals of 2 or 3 days, and 10 days after the last injection the animals were bled from ear veins to furnish sera for the complement fixation tests.

Complement Fixation Test.—The antisheep-cell system was used in the complement fixation tests. 0.2 cc. of diluted inactivated antiserum from rabbits that had received the different preparations of brain, 0.2 cc. of antigen (organ emulsions or extracts), and 0.2 cc. of guinea pig serum diluted so as to contain 2 units of complement, were mixed in a Wassermann tube and incubated for 30 minutes at 37°C, in a water bath. 0.2 cc. of 5 per cent suspension of washed sheep cells and 0.2 cc. (2 units) of antisheep-cell amboceptor were added and the tubes again incubated for 30 minutes at 37°C. The results of the tests were recorded in terms of the amount of fixation of complement, from 4+ indicating total complement fixation and no hemolysis to - designating complete laking of the sheep cells. Two types of antigen were used in the tests, aqueous emulsions and alcoholic extracts. order to make aqueous antigens 1 gm, of the particular organ desired was thoroughly ground in a mortar with alundum, and 25 cc. of physiological saline solution were added by increments during the process of grinding until a homegeneous emulsion resulted. This suspension was filtered through a tightly folded pad of absorbent cotton to remove the alundum and large bits of tissue. When less

concentrated emulsions were used, the necessary dilutions were made from the original 1:25 suspension. The preparation of alcoholic antigens was accomplished by the evaporation of a known volume of the alcoholic organ extract almost to dryness on a water bath and the suspension of the residue in a given volume of physiological saline solution. The concentration of this antigen was designated by the ratio between the original volume of alcoholic extract used and the amount of saline solution in which the residue was resuspended.

TABLE I

Summary of Results of Complement Fixation Experiments Indicating the Presence or Absence of Antibodies in the Sera of Rabbits Injected with Various Emulsions and Extracts of Homologous Brain

	Amount of complement fixed by different antisera in the presence of an aqueous emulsion of rabbit brain								
Material used for immunization			Dilution	of serum					
	1:5	1:10	1:20	1:40	1:80	1:160			
Fresh rabbit brain		_		_	-	_			
" " plus pig serum	4+	4+	2+	-		-			
Autolyzed rabbit brain	4+	4+	4+	4+	2+	_			
Vaccine virus rabbit brain	4+	4+	4+	4+	1+				
Alcoholic extract of rabbit brain plus pig serum	4+	4+	1+	-	_				

<sup>4+</sup> indicates complete fixation of complement.

The results given for alcoholic extract of rabbit brain plus pig serum were taken from another experiment and are therefore not strictly comparable to the others.

# Antigenicity of Homologous Brain

In order to test the antigenic qualities of homologous brain six experiments were performed, in which 79 rabbits were used. The animals in each experiment were divided into groups of four or five, and each group of animals received injections of one of the preparations of homologous brain tissue described above. For the most part each experiment was a repetition in whole or in part of the others. The results obtained in a typical experiment have been summarized in Table I and clearly show (1) that rabbits injected with fresh emulsions of homologous brain developed few or no antibodies against brain tissue, (2) that antibrain antibodies were elicited by fresh emulsions of

<sup>-</sup> indicates no fixation.

homologous brain to which pig serum had been added, (3) that autolysis alone changed the nature of homologous brain tissue to such an extent that it became a complete antigen, (4) that rabbits which received injections of emulsions of fresh homologous brain infected with vaccine virus readily developed antibrain antibodies, and (5)

TABLE II

Summary of Results of Complement Fixation Tests Conducted with Sera of Rabbits

Immunized with Autolyzed Homologous Brain Emulsions

					Dilution								
			Intigen		of antigen	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Emulsio	n of	rabbi	- it brai	n	1:50	4+	4+	4+	4+	4+	1+	3+	_
"	"	**	kidi	ney	1:50	4+	4+	3+	_	_	-		l
"	"	"	live	r	1:50	4+	1+			-	l I	l	l
"	"	"	sple	en	1:50	3+	_	_	_	l –		Ì	1
"	"	"	red	cells	1:25	_	_	-	-	_	_	l	
**	**	guin	ea pig	brain	1:50	4+	4+	4+	4+	4+	4+	1+	
"	**	"		kidney		4+	4-1-		_	_	_		
"	"	"		liver		4+			_	_	_		
**	**	44		spleen		4+	1 .	t -	۱ –	<u> </u>	_	1	1
"	"	"		red cells		_		<b> </b>	_	l _	_		Į.
Alcohol	ic ex	tract	of ral	bit brain	. 1:2	4+	4÷	3+	_	-	_	1	
44		"	"	" kidney	. 2:1		_	_		_	_	1	
"		64	**	" liver		<b> </b> _	_	_	_	_	_		1
**		"	"	" spleen		<b> </b> _	-	_	_	_	_		

<sup>4+</sup> indicates complete fixation of complement.

When aqueous emulsions were used as antigens in the tests lack of organ specificity is evident. Organ specificity is obvious in the tests in which alcoholic extracts were used.

that antibodies against brain tissue appeared in the serum of rabbits injected with emulsions of alcoholic extracts of rabbit brain to which pig serum had been added.

## Specificity of the Antiscrum

Having demonstrated that autolytic or disease processes acting on brain tissue can cause it to become fully antigenic for homologous animals, we proceeded to determine whether the antibodies elicited by

<sup>-</sup> indicates no fixation.

such altered brain material are specific for brain. For this purpose, an antiserum prepared in rabbits by means of injections of emulsions of autolyzed brain tissue was subjected to complement fixation tests in which aqueous emulsions of rabbit brain, kidney, liver, spleen, and red blood cells as well as emulsions of similar organs of the guinea pig were used as antigens. In addition, the antiserum was tested against alcoholic extracts of various rabbit organs. The results of one experiment are shown in Table II. The antiserum reacted most strongly with rabbit and guinea pig brain, yet some antibodies were also present

TABLE III

Summary of Results of Complement Fixation Tests Conducted with Sera of Rabbits

Immunized with Alcoholic Extracts of Rabbit Brain Plus Pig Scrum

			A	Dilution								
			Antigen	of antigen	1:5	1:10	1:20	1:40	1:80	1:160		
Emulsio	n of	abbi	t brain	1:50	4+	4+	4+	3+	1+	_		
**	ct	**	kidney	1:50	2+		_	_	-	-		
**	"	"	liver		`	_		_	-	_		
**	66	"	spleen	•	_			_	-	_		
c t	" ;	guine	a pig brain		4+	4+	4+	2+		-		
"	"	"	" kidney		3+		_		_	-		
44	"	"	" liver			_						
"	"	"	" spleen	, ,	1+		_	-		-		

<sup>4+</sup> indicates complete fixation of complement.

The results of the complement fixation tests show almost complete organ specificity. Compare with results shown in Table II.

against the tissues of practically all the other organs of the rabbit and guinea pig that were tested. When alcoholic extracts were used as antigens in the complement fixation tests, however, the antiserum was organ-specific (brain-specific) within the range of antigens used. Several workers (3, 6, 7, 8) have reported similar results for antisera prepared by the immunization of animals with emulsions of heterologous brain. Further tests for organ specificity were made on antisera which had been prepared by the injection into rabbits of alcoholic extracts of homologous brain plus pig serum. The results, summarized

<sup>-</sup> indicates no fixation.

in Table III, show that such sera were almost completely organ-specific. The minor fixations of complement in the presence of emulsions of other organs were overshadowed by the high titer of the antisera in the presence of brain antigen. This quantitative organ specificity of antisera prepared by injections of alcoholic extracts of brain has been noted by a number of investigators (9, 10).

When one considers the complex nature of the emulsions of brain with which the rabbits were immunized, a possible cause becomes apparent for the lack of specificity evidenced by the antibodies when they were tested for ability to fix complement in the presence of aqueous emulsions of different organs. The brain undoubtedly contains certain tissues which are common to other organs. For lack of more exact knowledge we shall consider the common factor to be connective tissue. Rabbits immunized with emulsions of brain therefore develop antibodies against connective tissue as well as against the specific brain antigen. Since the connective tissue antigen apparently is not as alcohol-soluble as is the antigen of brain, complement fixation tests done with alcoholic antigens show more pronounced organ specificity. If this reasoning is correct, the serum of rabbits immunized against emulsions of brain must contain at least two, perhaps more, kinds of antibodies, one specific for brain antigen and another which is not organ-specific. A similar theory for the lack of specificity in antisera prepared in animals by the injections of emulsions of heterologous organs has already been advanced by Fleisher and his co-workers (6, 7).

# Absorption of Antibodies

In order to determine whether non-specific as well as specific antibodies were present in the antibrain sera a number of absorption experiments were performed.

Method of Absorption.—A 1:25 emulsion, in physiological saline solution, of the desired organ was made in a manner similar to that used for the preparation of antigens for complement fixation tests. The technique of this procedure has already been described. The organ emulsion was then added to an equal volume of a 1:2.5 dilution, in physiological saline solution, of the antiserum, and the mixture was heated at 56°C, for 2 hours in the water bath. After standing overnight at room temperature it was centrifuged at high speed for half an hour, and the supernatant liquid was recovered with a pipette. In order to remove all of the

antigen-antibody complex, however, it was necessary to pass the mixture through a Seitz filter. As a control a second sample of the same antiserum was subjected to similar manipulations with the exception that physiological saline solution was added to it instead of an absorbing antigen. Experiments were also carried out in which an alcoholic extract of brain was used as an absorbing agent. The technique was similar to that just described except that a 1:16 dilution of the alcoholic antigens was found to yield maximum absorption.

TABLE IV

Summary of Results of Complement Fixation Tests Conducted with Antibrain Serum before and after Absorption with Aqueous Emulsions of Rabbit Kidney.

	Antigen			Dilution	Dilution of scrum							
Serum			Antige	n		of antigen	1:5	1:10	1:20	1:40	1:80	1:160
Unabsorbed	Emulsio " " " "	n of 	« « guine	kid live sple	ney er een brain	1:600 1:50 1:50 1:50 1:600	4+ 4+ 3+ 4+	4+ 1+ - 4+	3+ 3+ - 4+	1+ - - 2+	- - -	
	"	"	"		kidney liver spleen	1:50 1:50 1:50	4+ 4+ 4+	4+ 4+ 1+	2+ 1+ -	 	_	- -
Absorbed with aqueous emulsion of rabbit kidney	Emulsion	"	rabbi	kidr liver sple sple ii	ney r en	1:600 1:50 1:50 1:50 1:600 1:50 1:50	4+ - - 4+ - -	4+ - - 4+ - -	2+ - - 3+ - -	- - - 2+ - -	1 1 1 1 1 1	

<sup>4+</sup> indicates complete fixation of complement.

The results of the absorption experiments show that an emulsion of rabbit kidney removed the non-specific antibodies from the antibrain serum.

Three kinds of absorption experiments were conducted. In the first, antibrain sera were absorbed with emulsions of rabbit kidney which, containing only non-specific antigen, should not remove specific antibodies for brain. In the second, emulsions of rabbit brain which contain both the specific and non-specific antigens were used for absorption. In this case both kinds of antibodies should be removed from

<sup>-</sup> indicates no fixation.

the antisera. And finally antibrain sera were absorbed with alcoholic extracts of rabbit brain which contain only or principally the specific antigen and should therefore remove from the sera only the specific antibodies for brain. The results of these absorption experiments are summarized respectively in Tables IV, V, and VI. From the results

TABLE V

Summary of Results of Complement Fixation Tests Conducted with Antibrain Serum before and after Absorption with Aqueous Emulsions of Rabbit Brain

Serum	Antigen	Dilution of	Dilution of serum					
Serum		antigen	1:5	1:10	1:20	1:40	1:80	1:160
	Emulsion of rabbit brain	1:600		4+	4+	3+	2+	1+
	" " " kidney	1:50	4+	4+	3+	-	-	
ļ	" " liver	1:50	4+	1+	-	-	-	
	" " spleen	1:50	3+	-	-	-		-
Unabsorbed	" " guinea pig brain	1:600	4+	4+	4+	2+		
	" " " kidney	1:50	4+	4+	2+		_	_
	" " " liver	1:50	4+	4+		•	_	-
	" " " spleen	1:50	4+	1+	-	_	<b> </b>	-
	Alcoholic extract of rabbit brain	1:2	4+	4+	!	-	-	-
	Emulsion of rabbit brain	1:600	_		_	_	_	_
	" " kidney	1:50	<b>}</b> —	-		-	-	_
Absorbed	" " liver	1:50	-	_	-	_	_	
with aque-	" " spleen	1:50		_		-	-	-
ous emul-	" " guinea pig brain	1:600	-	l		_		_
sion of	" " " kidney	1:50	-	-	_	_	_	_
rabbit	" " " liver	1:50	-	-	_	-	-	_
brain	" " " splcen	1:50	_		_	_		_
	Alcoholic extract of rabbit brain	1:2	-	-	-	-	-	-

<sup>4+</sup> indicates complete fixation of complement.

The results of the absorption experiments show that an emulsion of brain removed both the specific and non-specific antibodies from the antibrain serum.

shown in Table IV it can be seen that an emulsion of rabbit kidney absorbed the non-specific antibodies from the serum so that it failed to react to any organ emulsion except that prepared from brain. When the absorption was carried out with an emulsion of rabbit brain (Table V) which contains both the specific and non-specific antigens, both kinds of antibodies were removed and the serum failed to fix comple-

<sup>-</sup> indicates no fixation.

ment in the presence of any of the organ emulsions. Finally, absorption of the antiserum with an alcoholic extract of rabbit brain (Table VI) resulted principally in the removal of antibodies against brain, permitting the serum still to react with emulsions of all the organs which contained the non-specific antigen. One might wonder why the antiserum absorbed with the alcoholic extracts of brain did not react to a slight extent with emulsions of brain as well as with emulsions of other organs because brain tissue contains both specific and

Summary of Results of Complement Fixation Tests Conducted with Antibrain Scrum before and after Absorption with Alcoholic Extract of Rabbit Brain

Serum	Alcon	holic Ext	taucted with Antibrain Scrum Tract of Rabbit Brain
	Antigen	Dilution of antigen	Dilution of serum
Unabsorbed	Alcoholic extract of rabbit brain Emulsion of rabbit brain  " " kidney  " " liver	1:2 1:600 1:50	1:5   1:10   1:20   1:40   1:80   1:160 4+
Absorbed with alco- holic ex- tract of rabbit brain	Alcoholic extract of rabbit brain  Emulsion of rabbit brain  "" kidney "" kidney "" liver "" spleen	1:50   4 1:2	- 2 - 1 - 1 - 1 -
4+ indicates - indicates The results	s complete fixation of complement.	1	

The results of the absorption experiments show that an alcoholic extract of brain removed principally the antibrain antibodies from the antibrain serum.

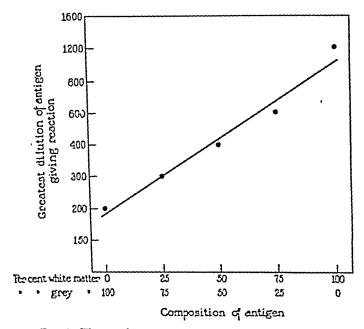
non-specific antigens. This is explained by the fact that in order not to mask the sensitivity of the complement fixation test by a large mount of antigen, it was necessary to use a 1:600 dilution of the emul-At this concentration the non-specific antigen contained brain tissue was diluted beyond its power to fix complement in the resence of the antiserum. The results of the absorption experiments st enumerated appear to indicate that two if not more antigen-antidy systems were involved, one of which was specific for brain while

<sup>-</sup> indicates no fixation.

the other was associated with some element or elements common to many organs.

## Nature of the Antigen

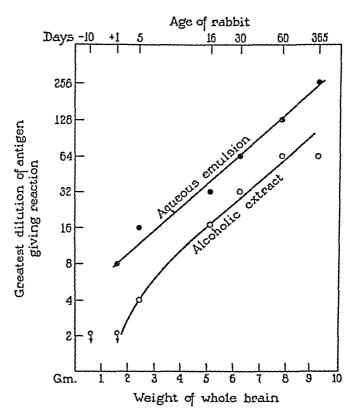
Having satisfied ourselves that emulsions of homologous brain can be made to become antigenic under certain conditions we were interested to determine what substance in the brain causes it to act as a specific antigen. It occurred to us that the specificity might be



TEXT-Fig. 1. The graph represents the relative antigenicity of white and grey matter of brain. The greatest dilution of each mixture of antigens which fixed complement in the presence of an antibrain serum has been plotted against the composition of the mixtures of white and grey matter.

associated in some manner with myelin. The most obvious way of testing such an idea was to find out whether the amount of myelin in brain antigens used for fixation of complement in the presence of antibrain sera runs parallel to the degree of fixation. A rabbit brain was therefore divided as nearly as possible into two portions, white matter which contains large numbers of myelinated nerve fibers and grey

matter which has a lower myelin content. From the two portions, aqueous emulsions were made, after which five mixtures were prepared by the combination of different quantities of them so that the ratio of grey emulsion to white emulsion varied from 100 per cent white to 100 per cent grey. The antigenicity of each of the emulsions was de-



Text-Fig. 2. The graph represents the relative antigenicity of brains of rabbits of different ages. The greatest dilution of antigen which fixed complement in the presence of an antibrain serum has been plotted as a function of the weights of the whole brain from which the antigen was made. The ages of the rabbits from which the brains were removed is also indicated. The ordinates for the curve for aqueous antigens are plotted as dilutions of a 1:50 brain emulsion; i.e., a 1:2 dilution in the graph is actually a 1:100 emulsion of brain tissue.

termined quantitatively in complement fixation tests conducted with antibrain serum. Text-fig. 1 shows the results graphically. It is apparent from them that the antigenicity of the white matter was roughly six times stronger than that of the grey matter, a property which at least paralleled the content of myelin in the two emulsions.

Further investigation of the relation between the myelin content and the antigenicity of brain tissue was made possible by the fact that myelin is practically absent from the central nervous system of fetal or newly born animals and increases with the age of the animal. We, therefore, prepared emulsions of the brain tissue of rabbits of different ages, ranging from the fetal stage to maturity. The relative antigenic properties of these emulsions were determined by the fixation of complement in the presence of antibrain serum. Furthermore, in order to eliminate as far as possible the non-specific antigen, alcoholic extracts were made from portions of the brains of these animals and tested for antigenicity. In Text-fig. 2 the results of such an experiment are shown graphically. It is immediately apparent that in newly born rabbits the content of organ-specific antigen in the brain emulsions was negligible and that it increased steadily with the age of the animal to reach a high concentration at maturity. Alcoholic extracts of brains of fetal or newly born rabbits failed to fix complement in the greatest concentration used, while extracts of brains of adult rabbits contained sufficient antigen to fix complement in high dilutions.

Observations on the Clinical and Pathological Findings in Rabbits Injected with Emulsions and Extracts of Homologous Brain

The rabbits which received injections of the different emulsions or extracts of brain were carefully observed during and after the course of immunization for the appearance of abnormalities attributable to the procedures used. In no instance was a reaction seen immediately following primary injection. For the most part the rabbits remained in good condition throughout the experiments, although some that received injections of emulsions of autolyzed brain lost weight during the course of the experiments. In every experiment, however, a certain number of rabbits developed signs referable to the central nervous system. The course in every case was the same: the rabbits developed a slight weakness of the hind legs which progressed rapidly to a complete paralysis of the hind quarters and was accompanied by a progressive emaciation; retention of urine was noted; and death occurred after an illness of a few days.

At necropsy the central nervous system and other organs in the gross were normal in appearance. In the brains of some of the paralyzed

rabbits stained sections showed perivascular infiltration and small areas of necrosis surrounded by zones of inflammation, in others evidences of healing lesions were seen, in still others no pathological changes were noted. Demyelination was not observed in any instance in the brain and cord, and the peripheral nerves appeared to be normal. Lesions identical in appearance with those just described were also found in some of the non-paralyzed rabbits sacrificed as controls and were considered to be due to the activity of *Encephalitozoon cuniculi*. The fact that the lesions were not present in all of the paralyzed animals and that they occurred in some of the non-paralyzed rabbits led us to conclude that they should not be looked upon as the

TABLE VII

Relation between the Number of Paralyzed Rabbits and the Immunizing Materials

Material used for immunization	No. of injections	No. of rabbits injected	No. paralyzed	Per cent paralyzed
Fresh rabbit brain		19 14	0	0 7
Alcoholic extract of rabbit brain plus pig serum	13-26 5-12	8 10 28	1 1 9	12 10 32

cause of the paralyses. This opinion is substantiated by the observations of Hurst (11), who, working with rabbits known to be free from *Encephalitozoon* infection, found no pathological changes in the central nervous system of animals which became paralyzed following injections of emulsions of heterologous brain.

It is interesting to note that the incidence of paralysis in the rabbits of the different groups paralleled the antigenicity of the brain emulsions and extracts that they received. In Table VII, the different immunizing emulsions and extracts are listed in the order of their antigenicity and the incidence of paralysis caused by them. None of the rabbits that received fresh emulsions of homologous brain, which has little or no antigenicity, became paralyzed, while 9 of 28 rabbits (32 per cent) injected with autolyzed homologous brain, a good antigen, showed evidences of paralysis. These observations, however,

cannot be considered evidence that the paralysis was directly related to the antigenicity of the brain emulsions, because Hurst (11) has pointed out that similar paralytic accidents follow injections of substances other than brain and also because the paralysis might have been induced by some toxic substance in the autolyzed or diseased brain tissue.

## DISCUSSION

It has been shown by a number of investigators that heterologous brain (2, 3), alcoholic extracts of heterologous brain plus pig serum (1, 10), homologous brain plus pig serum (4), and alcoholic extracts of homologous brain plus pig serum (10) excite in animals receiving them the production of specific antibrain antibodies capable of demonstration either by means of complement fixation tests or by precipitin reactions. In addition to this we have been able to demonstrate that homologous brain altered by autolysis alone or by infection with vaccine virus alone becomes antigenic and is then capable of inducing the production of antibodies specific for brain tissue. Furthermore, we have been able to show that the specific antigenicity of homologous brain runs parallel to the myelin content of the tissue.

In view of the facts just enumerated it is interesting to speculate about the etiology of certain diseases of the central nervous system, particularly the demyelinating maladies, such as the postinfection encephalitides, multiple sclerosis, Schilder's disease, and the encephalomyelitis occurring after antirabies vaccination. Speculation would be more interesting if it were possible to produce demyelination in animals by means of injections of brain tissue or if it were possible to demonstrate either brain antigen or antibrain antibodies in the blood of patients with demyelinating diseases. Hurst (11) from his review of the literature dealing with the effects on animals of repeated injections of brain tissue and from the results of his own experiments concluded that, although paralysis has been observed following injections of emulsions of brain, no one has definitely shown the presence of demyelination in the brains and cords of the paralyzed animals. Rivers,

<sup>&</sup>lt;sup>1</sup> After our work was completed, Lewis (13) reported that antibrain sera are also specific for testicular tissue. We did not examine our antibrain sera by means of complement fixation tests in which testicular tissue was used as an antigen. Consequently we have no information regarding this matter.

Sprunt, and Berry (12), however, recorded the fact that two of eight monkeys which received repeated injections of emulsions and alcoholic extracts of rabbit brain developed paralysis associated with demyelination. In regard to the presence of brain antigen or antibrain antibodies in the blood of patients with demyelinating diseases no significant reports have been seen. Although speculation about the relation of antibrain antibodies to certain demyelinating maladies is extremely intriguing, further facts must be obtained before a reasonable hypothesis concerning the matter can be presented.

### SUMMARY

Rabbits injected with fresh emulsions of homologous brain developed few or no antibodies capable of fixing complement in the presence of aqueous emulsions or alcoholic extracts of rabbit brain. Complement-fixing antibodies, however, were produced in rabbits by means of injections (1) of sterile emulsions of homologous brain which had been allowed to stand at room temperature for 5 to 30 days and (2) of emulsions of homologous brain experimentally infected with vaccine virus. The antisera that were produced following injections of emulsions of autolyzed homologous brain were shown by absorption tests to contain both specific and non-specific antibodies. The specific brain antigen was found to be approximately six times as abundant in the white matter as in the grey. It was almost absent from the brain of fetal and newly born rabbits, but increased in amount with the age of the animal to reach a maximum concentration at maturity. The specific antigen seemed to parallel the myelin content of brain tissue.

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# A THERMOPRECIPITATION REACTION IN TRYPANOSOMA EQUIPERDUM INFECTION IN LABORATORY ANIMALS

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Ascoli (1) showed that a thermostable substance associated with the anthrax organism would give a precipitate of diagnostic value, with the corresponding immune serum. Several other observers, among whom are Kraus (2), Piras (3), Philip and Hirst (4), Nai (5), Joos (6), Meuller and Tomcsik (7), Landsteiner and Furth (8), Dochez and Avery (9) and Zinsser and Parker (10) have found similar thermostable substances in bacteria and yeasts. The evidence points to the nature of these substances as carbohydrate. Since no such work has been done on trypanosomes, we decided to investigate T. equiperdum in regard to whether it possesses a thermoprecipitinogen.

## EXPERIMENTAL.

The precipitable substance was made as follows: Extracts of various organs and tissues of rats, guinea pigs and rabbits that were infected with T. equiperdum or had died from the infection, were tested by means of the precipitation reaction for the presence of a substance which reacts with the serum of recovered animals. Some of the organs and tissues tried were spleens, hearts, livers without the gall bladders, lymph nodes with their surrounding tissue, and blood drawn while the number of trypanosomes was as high as 1,670,000 per c. mm.

Extracts of each of these organs or tissues were prepared by cutting them into small pieces and then triturating them in the presence of talc. To this triturate was added five parts of physiological salt solution or water for each gram of the original organ or tissue. The suspension was then boiled for 5 minutes at 100°C. and permitted to cool without chilling. The liquid was separated from the residue of congulated protein and tale by centrifugalization. The supernatant fluid was

The extracts were generally prepared and used on the same day. 0.5 cc. of the clear extract was layered upon an equal quantity of immune serum in a small agglutination tube 100 x 11 mm. The results were read after 30 minutes at room

or body temperature and again after 18 hours in the ice box. Frequently tests which were negative or only weakly positive at the end of the 30 minute period of incubation showed a markedly positive reaction after standing 18 hours in the ice box. We have, therefore, recorded in table form only the readings after 18 hours in the ice box.

Within the last 2 years we have done thermoprecipitation tests on the extracts and sera of 108 white rats. 20 of these rats were not infected and the extracts and sera of these normal animals were used as controls. Of the other 88 infected rats, the organs tested were from animals in which the infection following its usual course had terminated fatally in from 4 to 6 days. In a few instances the animals were killed while being bled for immune sera. When this occurred during the 4th or 5th day of the infection, the organs were still used even though the normal course of the infection had been interrupted.

The serum used in each instance was obtained by letting the blood drawn from the normal or infected animals clot and pipetting it off aseptically after centrifugalization. It was not inactivated. In most instances pooled serum from several animals was used. The serum was kept in the refrigerator until ready for use. In this way the same lot of serum was used over a period of 2 or 3 weeks.

The tests were further controlled by using, not only the extracts of the whole blood of normal rats and others at the height of infection, but also the residue of trypanosomes and red blood corpuscles separated from the serum by centrifugalization. While there was an occasional weakly positive reaction with the red blood corpuscles and the trypanosome extracts, no positive reactions were observed with extracts prepared from the clotted cells and organisms which had been washed free of the serum before extraction.

The sera of rats that have been repeatedly infected with T. lewisi gave some precipitation reaction with spleen extracts of animals infected with T. equiperdum. In the same manner the immune sera of rabbits infected with T. equiperdum gave some precipitation reaction with spleen extracts of T. lewisi-infected rats. These group reactions were never marked but generally they were of a one plus or an occasional two plus reaction.

Within the last 12 months we have supplemented the test on the 108 rats with 39 guinea pigs, 10 of which were used as normals and the other 29 were infected with *T. cquiperdum*. The serum of the guinea pigs was generally taken during the 8th to 12th week of infection. The organs used for preparing the extracts were taken after death, which resulted from the infection or from the bleedings. Similarly 42 rabbits have been used in which 6 were controls, while the others were given mild infection. After the inoculation of small numbers of

living trypanosomes, some of the rabbits recovered from the infection within 12 to 16 weeks with only a slight evidence of having had the disease. If these animals were reinfected, they showed varying degrees of enhanced resistance to infection. The sera from the

## TABLE I

This table summarizes the results of thermoprecipitation tests when 0.5 cc. of clear extracts of the spleens of normal animals and animals infected with *T. equiperdum*, respectively, is carefully layered onto the surface of an equal amount of immune serum from various animals. All the results recorded represent readings after 18 hours in the ice box.

Extracts of spleen	Rat s	erum	Guinea p	oig serum	Rabbi	serum
	Normal	Immune	Normal	Immune	Normal	Immune
Normal rats, saline and aqueous	_	_	_	-		_
Infected rats, aqueous	-	+	<b> </b>	1 +	_	+++
Infected rats, saline	-	<b>±</b>	-	++	_	++
Normal guinea pigs, saline and aqueous	_	-		l –		_
Infected guinea pigs, aqueous	-		-	+	_	++
Infected guinea pigs, saline	_		-	<b>±</b>		+
Normal rabbits, saline and aqueous	l –	l –	l —	l –	l –	l –
Infected rabbits, aqueous	-	l –	-	+	_	1+++
Infected rabbits, saline	-	-	-	<u>+</u>	-	+++
T. lewisi-infected rats, aqueous and saline.	-	±	-	±	۱ –	+
T. lewisi-infected rats, aqueous and saline		ļ		1	1	l
extracts of red blood corpuscles and		-			1	1
trypanosomes	) —	-	) —	-	<b>)</b> —	) ±

<sup>+++,</sup> a gray ring of flakes 1 mm. or more in thickness at the junction of the two liquids, and sufficiently dense to be opaque when observed from above.

rabbits immunized in such a manner are the ones that showed the most pronounced reaction, generally given as three plus in Table I.

There were considerable variations in the reaction in each of the series of animals in which there were positive rings, but the table shows the predominating reaction for the combination noted.

<sup>++,</sup> a gray ring about 1 mm. in thickness at the junction of the two liquids but not sufficiently dense to cut off vision when observed from above.

<sup>+,</sup> a distinct gray line at the junction of the two liquids when observed from the side of the tubes, but transparent when observed from the top.

 $<sup>\</sup>pm$ , a faint gray line at the junction of the two liquids when observed from the side, but transparent when observed from the top.

When the spleen and liver extracts of guinea pigs dying of tuberculosis, and of rabbits dying from intravenous injection of Bargen's diplococcus were layered on equal volumes of the immune sera from the trypanosome-infected animals, the results were negative. These results were considered as additional controls.

When the organs or tissues of infected animals were extracted at room temperature for 8 hours they did not give more than a one plus reaction at any time.

We hope that the perfection of a test of this nature may be of value in giving an inexpensive test for the diagnosis of human, as well as of veterinary, trypanosominasis during the chronic or relapse stage when there are small numbers of trypanosomes in the peripheral circulation. Under these conditions the demonstration of trypanosomes is difficult by both the stain method and the dark-field examination.

## DISCUSSION

It is of interest to note that the precipitable substance does not appear to be within the trypanosomes themselves, but is present in certain organs, chiefly the spleen and liver in which there are large This is shown numbers of trypanosomes undergoing various changes. apparently by the negative outcome of the test with heart's blood of the rats containing 1 to 2 million trypanosomes per c.mm. or compared with the variously positive outcome of the test with extracts of spleens of the same animals similarly treated. This may mean that the thermoprecipitinogen resides in the splenic tissues and may be a product of the interaction between the trypanosomes and that part of the body The extracts of normal defense which is located in the spleen. spleens, as well as of the spleens and livers of animals infected with tubercle bacilli and Bargen's diplococcus, were negative, which is evidence in favor of the specific nature of the reaction in trypanosomal infections.

According to Hughes (11) Smith has shown that a thermolabile precipitable substance was present in the virus-free extracts of the testes of rabbits infected with a testicle-adapted strain of vaccine virus. Hughes also observed that the confirmatory evidence of Craigie and Tullock showed that the precipitable substance was not due to the organism *pcr sc*, but to a protein decomposition product

associated with the tissue reaction to the organism. This substance was not necessarily antigenic.

Except for the fact that the antigen is a thermostable substance, little is known as to its exact nature. We do know, however, that it is free from all protein that can be coagulated by heat, either in the presence of water or saline solution. It may conceivably be a polysaccharide with immunologically specific properties.

## SUMMARY AND CONCLUSION

There is a thermoprecipitinogenic substance in extracts of the spleen of rats, guinea pigs and rabbits infected with *T. equiperdum*. It does not appear to be within the body of the trypanosome itself.

Antibodies to this heat-resistant precipitable substance were found in the serum of infected animals.

The antibody strength seems to be relatively less in the serum of rats than in the other animals but the power of extracts from the spleen of infected rats appeared to be equivalent to the power of similar extracts of the other animals.

The antibody titer of the serum of rabbits was greater than in the case of the other two species investigated. This was shown not only by the reaction with the extracts of spleens of the same species, but also by the reaction with extracts of the spleens of similarly infected animals of other species.

I wish to express my appreciation to Dr. Claus W. Jungeblut of the Department of Bacteriology of the College of Physicians and Surgeons of Columbia University, whose preliminary criticisms of this article caused the control test with *T. lewisi* to be added.

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## A STUDY ON THE MECHANISM OF INVASIVENESS OF STREPTOCOCCI

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## PLATES 34 AND 35

(Received for publication, July 13, 1934)

Opie (1) and Menkin (2-6) have demonstrated that various materials are fixed in situ when injected into an acutely inflamed area, and Menkin has emphasized the rôle of mechanical obstruction in the form of a fibrinous network and thrombosed lymphatics in the fixation of such substances as bacteria and trypan blue as an important factor in the resistance of tissues to invasion. Recently, Menkin (6) has ascribed the characteristic lack of invasiveness of Staphylococcus aurcus to the irritating properties of that organism which induce a prompt inflammatory response as shown by the early fixation of trypan blue. On the other hand, the fixation of trypan blue in inflamed areas produced by Streptococcus haemolyticus was delayed about 45 hours, which led him to suggest that the greater invasiveness of this organism may be due to its lack of irritating properties and consequent delay in inflammatory response, thus leaving the lymphatics open as available routes for dissemination.

The experimental data set forth below indicate that the supposed paradox is illusory. Menkin's observations (6) are fully confirmed as to the behavior of staphylococcus and streptococcus in the skin of rabbits, but the delay of inflammatory fixation of trypan blue by streptococci probably is due to the production of two substances, one of which is fibrinolytic, while the other acts by inhibiting fibrin formation. Experimental evidence is given for the existence of these two substances, and certain of their properties are described. The effects of cultures and filtrates of certain distinct strains of Streptococcus kaemolyticus, Streptococcus viridans, and Staphylococcus aureus on the fixation

of trypan blue, and on fibrin formation in vitro, have been determined and compared with the relative invasiveness of the strains as determined experimentally.

## EXPERIMENTAL

Each experiment described has been repeated from two to ten times. The animals used were of mixed breeds and were purchased in the markets of Beirut as needed. All animals used weighed between 1,500 and 1,800 gm. Individual variations in reactivity of the animals to infection necessitated the many experiments. Those described below have been selected as being typical.

The organisms studied were grown in either Hartley's broth or dextrose (2 per cent)-phosphate-veal infusion broth. Comparable results were obtained with the two media, but the latter gave good growth more consistently, therefore it was used almost exclusively for the later experiments.

We believed that a comparative study of several strains of strepto-cocci and staphylococci which showed differences in serological or pathogenic properties, or both, would yield more information about their invasiveness than a more limited inquiry. For this reason, we have studied two strains of scarlatinal streptococcus, Streptococcus haemolyticus Strain Dochez, and Streptococcus haemolyticus Strain Griffith; one strain of erysipelas streptococcus, Streptococcus haemolyticus Strain E<sub>1</sub> Birkhaug; Streptococcus viridans Strain O from our laboratory; and Staphylococcus aureus Strain Wood, and Strain P from our laboratory. With the exception of the two strains from our own laboratory, all strains were obtained from the Lister Institute in the summer of 1933.

Fixation Time of Our Strains of Streptococci and Staphylococci

A series of experiments were carried out to determine the time required to produce inflammatory fixation of trypan blue at sites of inflammation induced by the strains of streptococci and staphylococci which were to be studied.

Method.—This was essentially the same as that used by Menkin (6) except that we greatly reduced the size of the injections with the aim of reducing trauma and other physical factors as much as possible. The fixation time was determined as follows: 0.5 cc. of an 18 to 24 hour broth culture of the organism was injected intracutaneously, by means of a fine 26G or 27G needle, into the extensor surface of the fore leg of a rabbit 2 or 3 cm. from the shoulder joint. A series of

rabbits were thus injected and at appropriate intervals the degree of inflammatory fixation was determined by injecting 0.4 cc. of a 1 per cent solution of trypan blue in saline into the center of the inflamed area. 1 hour after the injection of trypan blue, the rabbit was placed under ether anesthesia and the axillary lymph nodes dissected out and examined for the presence of the dye. In each case, a strip of skin was taken from the inflamed area, fixed in Zenker's fluid, and sections examined microscopically.

The experiment was controlled by injecting the same amount of dye into the normal skin, into the skin following 0.5 cc. of saline, and into the skin after the

				P	resence o	f dye i	n rodes					
Dura- tion of inflam- mation	Stref kaemely Doch	ticus	Strep. koemolyt Griffitl	cus	Strep kcemoly E:	dicus	Strej virida O	). :ns	Sta Gur Wo	En2	Sta cur F	E23
	S*	D	s	D	S	D	S	D	S	D,	S	D
hrs.												
1	± .	_	+	+	+++	+	+++	++	+	+	+	+
2	++++	+	++++	+	++	+	+++	+	+	-		<b> </b> -
4	+	+	+	\ <del>+</del>	++	+	++++	+++	-	-	<b>-</b>	-
8	+	++	++++	+	+	+	++++	+++		-	-	-
24	++++	+++	+++	++	+	++	++	++		<b> </b> -	-	-
36	\+++	+++	++	+	++	++	+	+	-	-	-	-
48	++	±	++	+	+	+	-	-	-	-	-	-

TABLE I
Fixation Time of the Strains Used

injection of 0.5 cc. of sterile broth, and subsequently examining the axillary nodes on that side for the presence of the dye.

Preliminary experiments indicated that the lymph nodes showed the presence of the dye more effectively than did lymph collected from their efferent vessels, so that only the nodes were used in taking the readings. Complete fixation was indicated by the failure of the dye to reach the nodes; absence of fixation was indicated by the intense staining of the nodes. The intensity with which the nodes were stained was recorded by means of the conventional plus and minus signs.

The results of the experiment to determine the fixation time of the strains used below are presented in Table I. The data obtained give complete confirmation of Menkin's observations (6). Staphylococcus

<sup>\*</sup> Column heads refer to superficial nodes (S) and deep nodes (D) of the axilla.

like cocci.

produces fixation quite early (1 to 2 hours), whereas streptococ delays fixation as long as 48 hours. One of the most significant this brought out by this experiment is the differences in response to different strains of streptococci during the earlier periods of the experiment. It appears that the Dochez and Griffith strains allow profixation, but subsequently break down or alter the barrier to the whereas Strain E<sub>1</sub> and our *Streptococcus viridans* prevent this experiment. These differences are explained by our *in vitro* stud given below.

Study of sections of skin removed from the inflamed area of e animal showed that where there was fixation of trypan blue, lymphatics were markedly dilated and regularly occluded with a de reticulum of fibrin (Figs. 1 and 2) in which leucocytes and cocci w frequently enmeshed (Figs. 4 and 5). It was observed that the car laries were also frequently thrombosed in the areas where fixation dye had been complete. Where there was no fixation the lymphat were patent (Fig. 3), frequently collapsed, and fibrin was scarce be in the lymphatics and in the tissue spaces. The mobilization of leu cytes was marked in inflammatory areas due to staphylococcus wit about 2 hours, but there was a striking absence of phagocytic cells all cases where streptococci had been injected, although infiltrati with mononuclears was apparent in streptococcal lesions of more th 24 hours duration. The absence of granulocytes in the streptococ lesions doubtless is ascribable to the selective action of streptococ leucocidin upon neutrophils, described by Gay and Oram (7).

The histological picture in the rabbits injected with the Doclard Griffith strains showed occlusion of the lymphatics in the ear periods of inflammation (1 to 2 hours). On the other hand, to lymphatics in the skin of rabbits which had received E<sub>1</sub> and Strept coccus viridans were usually patent during the earlier period. Lat after 6 to 8 hours, some fibrin was usually present in the lymphatic of rabbits receiving any of the strains which were studied, but occurred either as a very coarse reticulum (Fig. 6) which apparent would offer poor filtration and which frequently occupied but a portion of the lumen of the vessel, or in the form of a loose granular deposit (Fig. 7) which would be a poor barrier to either dye or small particles.

These observations support Menkin's thesis that fixation is primarily due to mechanical obstruction in the form of thrombosed lymphatics and a fibrin network in the tissue spaces of the inflamed area. Also, the correlation between lack of fixation and patency of the lymphatics appears to be established.

## Inhibition of Inflammatory Fixation by Streptococci

It was our desire to test an hypothesis that streptococci might be capable of actively inhibiting inflammatory fixation. Since it is well known that Staphylococcus aureus is often capable of bringing about the clotting of oxalated plasma in vitro without the addition of calcium, there is reason to suppose that the prompt inflammatory fixation demonstrated above is at least enhanced by the clotting factor. With this in mind, experiments were designed to demonstrate the ability, if any, of streptococci to inhibit inflammatory fixation induced by Staphylococcus aureus.

Method.—The action of whole cultures, filtrates, and saline suspensions was studied. Results obtained with the whole cultures and filtrates were essentially identical, while the saline suspensions gave but little evidence of activity within the time periods to which our observations were limited. 0.5 cc. of the strepto-coccus material to be tested was injected intracutaneously into the skin of the fore leg of a rabbit. 20 minutes later, 0.5 cc. of a saline suspension of Staph. aureus Strain P, in a concentration of about 5 billion cocci per cc., was injected into the same area. Since the strain of staphylococcus which was used always produced complete fixation in less than 2 hours (Table I), the degree of infiammatory fixation was determined, as described on page 5S2, at intervals of 2, 3, and 4 hours to ascertain any delay in fixation which might be attributable to the action of the streptococci.

A typical experiment would be: 9 a.m., 0.5 cc. of Strep. haemolyticus (Dochez) whole culture injected into right fore leg of a rabbit. 9:20 a.m., 0.5 cc. of Staph. aureus injected into same area. 11:20 a.m., 0.4 cc. 1 per cent saline solution trypan blue into same area. 12:20 m., axillary lymph nodes dissected out, rabbit under ether anesthesia. Presence of dye ascertained, and skin taken for microscopic examination.

In this manner, a comparative study was made of the inhibitory activity of the streptococcus strains indicated above. Results obtained with the whole cultures and filtrates were essentially identical, while the saline suspensions gave less evidence of activity within the time periods to which our observations were limited. Table II shows the result of a typical experiment in which streptococcus filtrates were used. Each of the strains of streptococcus used in the experiment was able to delay complete fixation for at least 4 hours, *i.c.*, more than twice the period of time required for the staphylococcus alone to produce complete fixation. Here again the time element indicates a difference in the mode of action of the different strains.

Histological examination of inflamed skin from these animals showed that, where lack of fixation was indicated by passage of the

TABLE II

The Influence of Streptococcus Filtrates on the Fixation of Trypan Blue in the Presence of Staphylococcus aureus

			D	uration o	f inflammat	ion*		`
Strain tested	1 hr		2 1	irs.	3 hr	s.	4 hr	3.
	S	D	S	a	s	D	S	D
Dochez	+	+	+	+	++++	++	++++	++
Griffith	++	+	++	+	++++	++	1++++	++
$\mathbf{E_i}$	++++	+++	++	+++	++	++	+	-
Strep. viridans	+++	++	+++	++	+++	++	+	_
Staph. aureus (C)	+	-	-	-	-	-	-	

<sup>\*</sup> The period indicated is the elapse of time between the injection of the staphylococcus and the injection of trypan blue.

dye, the lymphatics were more or less patent, although some fibrin was usually present in the lumina of the vessels. The presence of the streptococcus filtrates suppressed the granulocyte infiltration, even in the presence of staphylococcus, indicating that the lack of these cells in streptococcus lesions is not necessarily due to lack of stimulating substances.

We conclude from the results of the experiments described in this section that the lack of fixation in an area of inflammation induced by streptococcus is due to the production by these organisms of a substance or substances which actively inhibit fixation.

## Relation of Inhibition of Fixation to Invasiveness

The demonstration of the active rôle of streptococci in the inhibition of inflammatory fixation of dye suggested that the invasiveness of these organisms might be due to, or dependent upon, the production of inhibitory substances. The following experiment was planned to demonstrate the relative invasiveness of our strains, and to allow comparison of the results with the data given in Table I.

Method.—24 hour dextrose-phosphate broth cultures of the strains to be tested were centrifuged in Hopkins' tubes at high speed, and the number of organisms in the cultures determined. The cocci were then resuspended in a sufficient quantity of the supernatant culture fluid to give a suspension of 1 billion cocci per cc. Of such a suspension, 0.5 cc. (500 million organisms) was injected intracutaneously into the fore leg of each of a series of normal rabbits. At intervals of 30 minutes, 1 hour, 2, 3, 4, 8, and 24 hours, one rabbit of each series was anesthetized with ether, and all superficial and deep axillary lymph nodes were carefully dissected out with aseptic precautions. The superficial and deep nodes were placed separately in sterile Petri dishes, and immediately taken to a "sterile room" where they were thoroughly ground with sterile sand by means of sterile mortars and pestles. The macerated nodes were then suspended in 5 cc. of sterile saline and two blood agar pour plates made, using 0.5 cc. and 0.05 cc. of the suspension respectively. This method gave two pour plates for each group of regional or deep nodes. The plates were incubated at 37°C. for 48 hours, and the number of hemolytic colonies determined. From these data, the approximate number of organisms present in the lymph nodes was then computed. The experiment has been repeated with remarkably little divergence in results considering the degree of probable error inherent in the method.

The results of this experiment are given in Table III, which clearly shows the relative abilities of the strains under consideration to pass from the site of injection to the lymph nodes in given periods of time. Comparison with data on the fixation of trypan blue shows that there is a significant degree of correlation between the number of organisms and the amount of dye in the lymph nodes at the end of corresponding periods of inflammation due to homologous strains. The differences in the behavior of the various strains as shown by the bacterial counts and the passage of trypan blue are notably parallel.

We conclude from this experiment that invasiveness by the bacteria and the inhibition of inflammatory fixation of trypan blue are correlative.

TABLE III Determination of Relative Invasiveness\*

						I	Duration of inflammation	of inflamı	nation					
Strain tested	30 min.	nin.	1 hr.	H.	2 hrs.		3 hrs.	rs.	4 hrs.		8 hrs.	p <sup>*</sup>	24 hrs.	ž,
	S	Ω	S	D	S	Ω	S	Ω	S	Д	S	D	S	Q
Dochez	ا ۶	01	0	01	100	100+	150 +	0+	0+	<b>a</b> +	0+	10 ++	7,000 200	200 +++
Griffith	270	0+	4,800	240	24,000	٠ +	0+	0+	30	0+	+++++	0+	30	0+
Eı	800	0+	720	78	<del>4</del> +	0+	0 1	0	009	130	0+	0+	0+	90 +
Strep. viridans			30	20 ++	0 +	0++	160	0 ++	40++++	+++	40 0 1,300 200 ++++ +++++++++++	200 +++	1,700	300
Slaph, aureus	0+++	0 +	0+	0+	0	0	01	0 1	30	0	25	0	01	01

The numerals represent the number of cocci recovered from the The plus and minus signs indicate the quantity of dye capable of reaching the lymph nodes at the end of corresponding periods of inflammation, introduced for comparison. lymph nodes, indicated as S, superficial, and D, deep. \* 500 million organisms were injected in each case.

In Vitro Studies. Influence of Streptococci on Fibrin Formation

In vitro studies were undertaken to see if they would yield additional information about the mechanism by means of which our strains of streptococci are able to get past the barrier of inflammatory fixation. This work was just begun when Tillett and Garner (8) described the production of a fibrinolysin by virulent hemolytic streptococci of human origin, and it immediately occurred to us that the explanation of our in vivo results lay in the phenomena described in their paper. However, Tillett and Garner had found that their fibrinolytic strains were incapable of acting upon rabbit plasma clot, hence their data could not be applied to our results without reinvestigation. We undertook to compare the action of our strains of streptococci upon both human and rabbit plasma.

Method.—The method of choice was the one described by Tillett and Garner (8), using oxalated human and rabbit plasma. This method uses 0.2 cc. of plasma plus the material being tested (filtrate or whole culture) and saline to give a total volume of 1.5 cc. After thorough mixing, 0.25 cc. of a 0.25 per cent solution of calcium chloride is added to the tube and the mixture vigorously agitated for a few seconds. The tube is immediately placed in a water bath held at 37°C, and kept under observation for 24 hours. Control tubes containing only plasma. saline, and calcium chloride are used for each series of experiments; the controls usually clot in 5 to 7 minutes. The human plasma used in our experiments was always obtained from two individuals whose fibrin clot was susceptible to lysis. When rabbit plasma was used, blood was obtained from several normal animals by bleeding from the heart, and their plasmas were pooled before use. More than 50 experiments have been carried out in the course of our in citro studies. In our experience, dextrose phosphate broth containing 2 per cent dextrose gave more active cultures and filtrates than did the medium recommended by Tillett and Garner (8) which contained but 0.5 per cent dextrose.

The results of a typical series of experiments are given in Table IV, in which the influence of streptococcal filtrates upon both human and rabbit plasma is compared. It can be seen from this table that there are two different types of action; namely, (1) fibrinolysis of human plasma clot associated with greatly accelerated retraction of rabbit plasma clot by our Dochez and Griffith strains, and (2) complete inhibition of coagulation by our E<sub>1</sub> and Streptococcus viridens strains. The Dochez strain has never shown any tendency to inhibit primary clot formation, and the Griffith strain inhibited coagulation on but

one occasion, when there was a very heavy growth in the culture. Our  $E_1$  strain has regularly produced both the inhibitory and the lytic factors, but usually the inhibitory factor masks the lytic one which is brought out only by dilution of the filtrate (Table IV). We

TABLE IV

The Influence of Streptococci on the Coagulation of Human and Rabbit Plasma

g	Amount	)	Human plasma		Rabbit plasma	
Strain tested	of filtrate	Coagula- tion time	Liquefaction time	Coagula- tion time	Liquefaction time	
	cc.					
Dochez	0.5	13 min.	2 hrs., 6 min.	10 min.	Retracted 3 hrs.	
ee	0.4	13 "	2 " 6 "	10 "	" 18 "	
· · ·	0.3	13 "	2 " 15 "	10 "	" 24 "	
"	0.2	13 "	2 " 30 "	10 "	Partial retraction	
	0.1	13 "	8 "	10 "	66 66	
Griffith	0.5	11 "	1 hr. 4 min.	10 "	Retracted 2 hrs.	
"	0.4	11 "	1 " 30 "	10 "	" 18 "	
"	0.3	11 "	1 " 33 "	10 "	Partial retraction	
"	0.2	11 "	1 " 37 "	10 "		
"	0.1	11 "	1 " 45 "	10 "	" "	
$\mathbf{E_t}$	0.5	*			_	
u .	0.4			-	_	
c (	0.3	49 min.	18 hrs.	-	_	
CC .	0.2	16 "	20 "	32 min.	Retracted 24 hrs.	
"	0.1	10 "	Retracted 4 hrs.	10 "	" 24 "	
Strep. viridans	0.5	*		_		
u	0.4	-	-	_	-	
"	0.3	-	_	_	<del></del>	
· ·	0.2		-	_	-	
"	0.1	16 min.	Retracted 24 hrs.	20 min.	No change in clot	

<sup>\*</sup> Minus sign indicates failure to coagulate. Control tubes showed complete coagulation in 5 to 7 minutes.

believe that the possibility of the two phenomena, lysis and inhibition of coagulation, being simply quantitative manifestations of a single factor is obviated by the fact that our Dochez and Griffith strains have not characteristically produced inhibition, even when their quantity has been increased in the tube, and also by the fact that occasionally we have had E<sub>1</sub> cultures in which the inhibitory factor was lacking but in which the lytic factor was as active as that of our Griffith strain. The conditions controlling the appearance of the fibrinolytic and antifibrinogenic (inhibitory) agents have not yet been studied intensively by us.

The fibrinolytic substance which we have studied is undoubtedly identical with the one described by Tillett and Garner (8), but the inhibitory factor described above has not been previously reported. We believe that the inhibitory phenomenon was missed by Tillett and Garner because of the medium in which they cultured their organisms as routine. In our experience, filtrates or whole cultures for which their medium was used failed to give any inhibition of coagulation, while corresponding cultures of Strain E<sub>1</sub> and Streptococcus viridans grown in phosphate-buffered broth containing 2 per cent dextrose were active in inhibiting clot formation.

In the course of our experiments we have also tested the  $E_2$  and  $E_3$  strains of *Streptococcus haemolyticus* var. *erysipelatis* for the fibrinolytic and antifibrinogenic factors. Both of these strains are unlike Strain  $E_1$  in that they are primarily fibrinolytic and have not shown any inhibition of clot formation.

On the whole, the appearance of the lytic and inhibitory factors in 24 hour dextrose-phosphate broth cultures of *Streptococcus liaemolyticus* and our *Streptococcus viridans* is quite regular. However, occasionally these substances may occur in but small amounts, or may even be absent. These irregularities are always associated with scanty growth of the organisms.

## Resistance of Fibrinolytic and Antifibrinogenic Factors to Heat

Preliminary experiments indicated that both the lytic and the inhibitory factors are relatively thermostable, so that only the critical series of experiments to determine this point are described here. Active filtrates of fresh 24 hour dextrose phosphate broth cultures were held in a water bath at 100°C. for periods of 20 minutes, 40 minutes, and 60 minutes; 0.5 cc. of each heated filtrate was then tested against human plasma.

The results of this experiment are given in Table V. Both types of substances are thermostable, requiring boiling for 1 hour to ac-

complish complete inactivation. However, the fibrinolytic substance is markedly weakened when boiled for ½ hour, while the antifibrinogenic substance is not noticeably affected until it has been heated for at least an hour. This difference in thermostability supports the evidence given above to substantiate the view that the two substances are essentially different. The degree of thermostability exhibited by the fibrinolytic and antifibrinogenic substances obviates the possibility of their being enzymatic in nature, and places them with the known streptococcal dermatotropic toxins in respect to their resistance to heat.

TABLE V

The Influence of Heat upon the Fibrinolytic and Antifibrinogenic Factors of Active

Streptococcus Filtrates\*

Strain tested		ated (C)	Heated	20 min.	Heated	40 min.	Heated 60	min.
	C.T.	L.T.	C.T.	L.T.	C.T.	L.T.	C.T.	L.T.
Dochez	8 min.	1 hr., 34 min.	10 min.	16 hrs.	7 min.	24 hrs., inc.	7 min.	0
Griffith	11 "	52 min.	10 "	12 "	7 "	24 hrs., inc.	7 "	0
$\mathbf{E_1}$	0	0	0	0	0	0	1 hr., 35 min.	0
Strep. viridans	0	0	0	0	0	0	1 hr., 50 min.	0

<sup>\*</sup> The filtrates were held in a water bath at 100°C., and tested in vitro. 0.5 cc. of filtrate was tested in each case. Column heads C.T. and L.T. indicate coagulation time, and time required for complete lysis (lytic time) respectively. The unheated filtrates (C) acted as controls.

Neutralization of Fibrinolytic and Antifibrinolytic Substances by Antisera

Our studies on the immunological aspect of the problem have not been completed, so that only brief mention of certain of our results will be made here. Tillett, Edwards, and Garner (9) have recently given convincing clinical evidence of the antigenic nature of streptococcal fibrinolysin, and have demonstrated that the plasma clot of a patient suffering from acute streptococcal infection becomes resistant to lysis coincident with the clinical changes leading to recovery.

Type-specific antisera prepared by immunizing rabbits against Dochez, E<sub>1</sub>, E<sub>2</sub>, and E<sub>3</sub> strains of Streptococcus haemolyticus have

hown certain protective properties, but a number of irregularities have necessitated further study. 0.1 cc. of type-specific Dochez antiserum has completely neutralized the fibrinolysin in 0.5 cc. of altrate of both the Dochez and Griffith strains. Type-specific. E<sub>1</sub> antiserum in amounts of 0.2 cc. or more has allowed plasma clot formation in the presence of 0.5 cc. of E<sub>1</sub> filtrate, but it has had no neutralizing effect on the fibrinolysin present in the same filtrate. None of the antisera tested so far have shown any neutralization of the antifibrinogenic factor produced by our strain of Streptococcus paridans.

Two commercial antistreptococcal antisera have been tested. These antisera were the products of the Bayer-Meister-Lucius-Behringwerke, Leverkusen, who kindly supplied us Scarlat-Streptoserin and Streptoserin. The former was a polyvalent antiscarlatinal serum; the latter was a polyvalent product recommended for all streptococcal infection. Both products have shown complete neutralization of both Dochez and Griffith fibrinolysin in amounts of 0.1 cc. of antiserum against 0.5 cc. of active filtrate. However, neither of these products neutralized the antifibrinogenic substances produced by our strains of E<sub>1</sub> or Streptococcus viridans.

A full report of our study on the immunological aspect of this problem will be published later. It appears that the testing of antisera against the action of active streptococcal cultures or filtrates on plasma clot offers a presumptive test of the anti-invasive properties of antistreptococcal sera prepared for therapeutic purposes. However, the development of an accurate test along this line must await the purification of either the antigen or the antibody for the establishment of a standard of potency.

## DISCUSSION

The essence of the concept of inflammation is that it is primarily an immediate local defensive or protective reaction of the tissues to the stimulus of an irritant. Although there is considerable overlapping, three fundamental phases are generally recognized as occurring in more or less definite sequence in acute inflammation. These phases are (1) exudation and coagulation of plasma proteins in the tissue spaces and lymphatics. (2) mobilization and phagocytic activi-

ties of leucocytes, and (3) repair. Menkin has recently reviewed the literature on the first two phases in detail, but for purposes of comparison the essential phenomena are summarized below in their chronological order. We are not concerned with the third phase, repair; but to appreciate the aptitude with which the invasive streptococci pass the local barriers, it will be necessary to consider the first two in some detail.

The first phase of an acute inflammation was vividly described by Cohnheim (10) who demonstrated the early dilatation of the blood vessels and capillaries, the exudation of plasma fluid, and the emigration of leucocytes from the vessels into the adjacent tissue spaces. Menkin (2) has demonstrated the early coagulation of the plasma proteins and thrombosis of the lymphatics. Our own observations include the early occlusion of some capillaries as well as lymphatics. Menkin has emphasized the rôle of the resulting fibrin barrier. If the irritant is bacterial, agglutinins, precipitins, and bactericidins present in the exuded lymph must undoubtedly facilitate the filtering action of the fibrin barrier in limiting dissemination. Inflammatory fixation appears to be the first line of defense, once the invaders have gained the tissues.

The second phase of acute inflammation is dominated by the appearance of leucocytes. These phagocytic cells are of two fundamentally different types, the granulocytes and the clasmatocytes, and although overlapping to a considerable extent, appear in a definite sequence in a sterile inflammation (11). By the time that fibrin formation is well under way, polymorphonuclear neutrophils appear in the inflamed area and within 3 or 4 hours they accumulate in These actively phagocytic cells which are so enormous numbers. readily mobilized via the blood stream are important defense factors against bacteria because of the speed with which they can be concentrated, and in the absence of an overwhelming invasion are capable of dispatching most of the invaders. We may consider the mobile army of neutrophils as the defenders of the first barrier of defense. However, after the inflammation has progressed for about 24 hours, there appear in increasing numbers a more slowly mobilized class of reserves from the tissues—the clasmatocytes—to consolidate the defense if necessary, or if the neutrophils have failed to stem the tide

of invasion they may assume the brunt of the defense (11, 12). Once past the inflammatory barrier, invasive organisms may be filtered out of the lymphatics by the regional lymph nodes, but their chances of gaining the blood stream directly or from the nodes are greatly enhanced.

If we consider the demonstrated properties of invasive streptococci in relation to the outlined aspects of resistance, the problem of the virulence of these organisms is greatly illuminated. The greater part of this paper has been devoted to the demonstration of how streptococci may negotiate the barrier of inflammatory fixation, probably by virtue of their fibrinolytic and antifibrinogenic properties.

The second phase of the inflammatory defense is apparently the critical period in determining the degree of limitation of invading organisms, and the frequent success of streptococci, particularly Streptococcus haemolyticus, in passing the barrier is readily explained by the production of streptoleucocidin by invasive strains (13, 14). This bacterial toxin is highly specific for the neutrophilic leucocytes, and with the slower mobilization of the resistant clasmatocytes, there must usually be a period of several hours during which the streptococci are relatively freely disseminated through the tissue spaces and lymphatics. Such an interpretation, with the demonstration of the inhibition of fixation by streptococci, (1) supports the claims of Gay and Morrison (11) that resistance to Streptococcus haemolyticus is primarily dependent upon successful mobilization of clasmatocytes: (2) explains the results of Gay and Clark (12) which indicated that polymorphonuclear leucocytes were relatively inactive in increasing the resistance of rabbits to their streptococcus; and (3) brings the results of Gay and his coworkers into harmony with those of other investigators who have emphasized the significance of the earlier periods of inflammation in resistance, or who were dealing with organisms other than Streptococcus kacmolyticus.

Streptococci manifest their virulence in two ways; namely, by the action of more or less specific histotropic toxins, and by their invasiveness. Both aspects of virulence are not necessarily manifested by the same strains, since highly toxigenic strains may show relatively little tendency to invade the tissues. Our results reported above suggest that lack of invasiveness is probably attributable to inability

of the streptococcus to elaborate either fibrinolysin or the inhibitory factor. Those strains capable of producing fibrinolysin should be able to invade and cause more serious conditions, such as septicemia, more readily than those strains which produce only the inhibitory substance. In the latter case the initial degree of invasion would be more dependent upon the size of the inoculum and the quantity of inhibitory substance introduced with the cocci, and it seems unlikely that much, if any, of this substance would be present under natural conditions of infection. The strains, such as the erysipelas strains, which produce both substances appear to be the best equipped for the invasion of tissues. The fact that the *Streptococcus viridans* strains which we have studied produce only the antifibrinogenic substance, or none at all, seems to us to be the most logical explanation of the characteristic lack of invasiveness of the *viridans* group of streptococci.

Our experimental observations are in agreement with the clinical experience that virulent hemolytic streptococci never produce a thick fibrinous pus; the pus produced is thin and free of fibrin threads. Also, our results suggest that the scarcity of polymorphonuclear leucocytes at the site of an inflammation due to virulent streptococci may be attributable to the absence of fibrinous reticulum which otherwise would retain the leucocytes *in situ* whether they were living or had been killed by leucocidal substances.

## SUMMARY

- 1. Menkin's observations of the failure of inflammatory fixation in areas of acute inflammation due to *Streptococcus haemolyticus* have been confirmed.
- 2. The lack of inflammatory fixation in the presence of streptococci is not due to the passive nature of the streptococcus, but may be attributed to the production of (1) fibrinolytic, and (2) antifibrinogenic substances which dissolve the fibrin barrier, or prevent its formation, thus maintaining the patency of the lymphatics and capillaries and facilitating the dissemination of the organisms.
- 3. The production of fibrinolytic or antifibrinogenic substances, and the invasiveness of a given strain of streptococcus are correlative.
  - 4. Both substances are relatively thermostable. Fibrinolysin is

destroyed if held at 100°C. for 1 hour. The antifibrinogenic substance is weakened but is not destroyed under the same conditions.

- 5. There is evidence that both substances are antigenic, and exhibit some degree of type specificity.
- 6. The rôle of fibrinolysin and the antifibrinogenic factor in the invasion of the tissues by streptococci is discussed.

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### EXPLANATION OF PLATES

All tissues fixed in Zenker's acetic fixing fluid, and stained by the Gram-Weigert method. Photographs taken with the aid of Zeiss apochromatic objectives and Leica camera.

### PLATE 34

- Fig. 1. Photomicrograph showing a densely occluded lymphatic and marked perilymphatic infiltration with granulocytes in the marginal area of an acute local inflammation induced in the skin of a rabbit by Staphylococcus aureus (Strain P). Duration of inflammation, 4 hours. × 200.
- Fig. 2. Photomicrograph showing the occlusion of the lymphatics in the skin of a rabbit, 2 hours after the injection of a suspension of Staphyloceccus aureus (Strain P).  $\times$  100.
- Fig. 3. Photomicrograph showing patent lymphatics and paucity of leucocytes and fibrin in an area of inflammation due to *Streptececcus haemelyticus* (Strain Griffith). Duration of inflammation, 4 hours. × 150.

### PLATE 35

Figs. 4 and 5. Photomicrographs of occluded lymphatics, showing the finer structure of the fibrinous reticulum and its role in retaining cocci in situ. Inflamma-

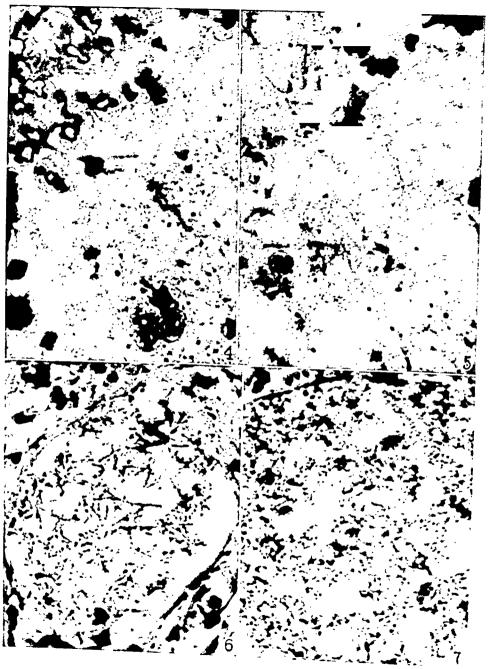
tion induced by Staphylococcus aureus (Strain P). Duration of inflammation, 4 hours.  $\times$  900.

- Fig. 6. Photomicrograph showing the loose reticular nature of fibrin in the lymphatics in an area of skin which had received 0.5 cc. of an 18 hour culture of Streptococcus hacmolyticus (Strain Dochez). Duration of inflammation, 6 hours. Contrast with Fig. 4.  $\times$  600.
- Fig. 7. Photomicrograph showing the loose, granular nature of the contents of a lymphatic vessel in an area of skin which had received 0.5 cc. of an 18 hour culture of *Streptococcus haemolyticus* (Strain  $E_1$ ). Duration of inflammation, 4 hours.  $\times$  900.



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## CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

IX. THE SPECIFICITY OF ANTIGENS PREPARED BY COMBINING THE p-Aminophenol Glycosides of Disaccharides with Protein

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(From the Hospital of The Rockefeller Institute for Medical Research) (Received for publication, August 9, 1934)

As a result of the work of numerous investigators a striking advance has been made during the past decade in our knowledge of the chemical and immunological properties of that unique class of naturally occurring substances, the specific bacterial polysaccharides. Complex carbohydrates possessing immunologically specific properties have been found widely distributed in many varieties of different microorganisms. The significance of these findings lies in the fact that these specific components, having in common the chemical properties of polysaccharides, are known to function as determinant substances in many of the specific reactions of bacterial infection and immunity. The fact that these carbohydrates, so closely related in chemical properties, remain sharply differentiated in biological specificity may eventually be explained on the basis of certain subtle differences in the chemical structure of the molecules that constitute these substances. Possible differences in stereochemical structure and in the position of intermolecular linkages between the individual sugars comprising the polysaccharides are manifold. Indeed, it is not unlikely that structural differences of this character account for the wide variation in the immunological specificity exhibited by carbohydrates of diverse origin.

It is already known that differences in the stereochemical structure of the carbohydrate radical in artifically compounded hexoside-protein antigens influence their immunological specificity (1). It has been shown that the azophenol glycosides of glucose and galactose, irrespective of the protein with which they are combined, give rise in the

animal body to the formation of antibodies which are distinct and specific for the carbohydrate radical in the antigenic complex. Although the difference in chemical structure between the glycosides of glucose and galactose is confined to an interchange of the H and OH groups on the fourth carbon atom of the hexose radical, yet this slight alteration in chemical configuration suffices to determine the biological specificity of antigens containing these hexosides. If, on the other hand, the structural differences in monosaccharides be confined to the carbon atom bearing the aglucon, as in antigens containing  $\alpha$ -and  $\beta$ -azophenol glucosides, then the immunological reactions of these two derivatives of glucose, though predominantly type-specific, show a certain degree of crossing, presumably attributable to the identity in structure of the five terminal carbon atoms of both glucosides (2).

Thus it has been established experimentally that the stereochemical configuration of the hexose radical has an important influence in determining the biological specificity of artificial antigens containing simple monosaccharides. Whether stereochemical changes in the hexose constituents of more complex saccharides exert a similar influence, and whether the position of linkage of hexose to hexose affects the course of antibody response, are questions of importance in elucidating the factors which govern the specificity of the reactive polysaccharides of bacterial origin.

The disaccharides are substances of known chemical constitutions, and hence are excellent derivatives to use in studying the influence of changes in intermolecular linkage and stereochemical structure on biological specificity. For this purpose, therefore, the p-aminophenol glycosides of lactose, gentiobiose, cellobiose, and maltose have been synthesized (3). The diazonium derivatives of these glycosides have been combined with protein and the resulting conjoined disaccharide-proteins have been utilized as antigens in the preparation of immune rabbit sera. The present paper is an account of the immunological properties of these artifically conjugated antigens. For purposes of comparison there are also included the serological properties of antigens containing monosaccharide derivatives prepared by combining with the same protein the p-aminophenol glycosides of  $\alpha$ - and  $\beta$ -glucose and of  $\beta$ -galactose.

#### EXPERIMENTAL

1. Methods.—The methods of preparing the mono- and disaccharide immunizing antigens used in the present study differ in no essential respect from those previously described (2 b). The method of intravenous immunization of rabbits, and the technique of the precipitin and inhibition tests, are the same as those employed in earlier studies. The immunizing antigens were prepared by combining each of the carbohydrate derivatives with the globulin of normal horse serum. In order to avoid the reactions of a common protein, the test antigens were similarly prepared by combining the same glycosides to the protein of chicken serum.

For purposes of brevity the p-aminophenol glycosides of the different sugars will be frequently referred to throughout the text and tables by the following abbreviations:  $\alpha = \alpha$ -glucoside;  $\beta = \beta$ -glucoside;  $\beta = \beta$ -glucoside;  $\beta = \beta$ -glucoside; and  $\beta = \beta$ -glactoside. The immunizing antigens prepared by combining these glycosides with globulin are referred to as  $\alpha$ -globulin, C-globulin, etc., and the corresponding antisera respectively as  $\alpha$ -antiserum, C-antiserum, etc. The test antigens, prepared by combining the glycosides with chicken serum protein are designated as  $\alpha$ -test antigen, C-test antigen, etc.

## I. Specific Precipitin Tests

1. Homologous Precipitin Reactions of Mono- and Disaccharide Antisera.—The sera of rabbits immunized respectively with antigens containing the three monosaccharide and the four disaccharide glycosides combined with horse serum globulin were first studied for the presence of homologous precipitins. Test antigens containing the same glycosides combined with the protein of chicken serum were used in the precipitation tests. The results of these tests are given in Table I.

From the results presented in Table I it is seen that antigens containing either mono- or disaccharide radicals give rise in each instance to immune bodies which react with the same carbohydrate derivative irrespective of the protein to which it is attached. The glycoside-proteins function as effective antigens in the animal body, and it is suggested that the large number of polar (OH) groups in the carbohydrate radical may account for this fact.

2. Heterologous Precipitin Reactions of the Disaccharide Antisera.—In order to ascertain whether the disaccharide antisera cross-react with test antigens containing the other disaccharides, each serum was tested for the presence of heterologous precipitins.

The results of the cross-precipitin tests are summarized in Table II.

From the results given in Table II, it can be seen that an antiserum prepared by immunization with C-globulin contains antibodies which precipitate not only the homologous C-test antigen, but the heterologous Ge-test antigen as well. Similarly the antiserum to Ge-globulin contains precipitins for the homologous test antigen and cross-reacts with the heterologous C-test antigen. Neither of these antisera, however, reacts to any appreciable extent with test antigens con-

TABLE I

Homologous Precipitin Reactions of Mono- and Disaccharide Antiscra\*

Antisera prepared by	Test antigenst		Final dilution	of test antigens	3
immunization with:		1:5,000	1:10,000	1:20,000	1:40,000
α-Globulin β " Ga " C " M " Ge " L "	α Chicken serum β " " Ga " " C " " M " " Ge " " L " "	+++± ++++ ++++ ++++ ++++ ++++	+++± ++++ ++++ ++++ ++++	++± +++ ++± +++± ++++ +++	++ +++ ++ +++ +++ ++± ++±

<sup>++++</sup> = complete precipitation with compact disk formation.

taining M or L. The M-antiserum, on the other hand, precipitates not only M- but also Ge- and C-test antigens; the M-antiserum does not react with test antigen containing L. The L-globulin antiserum precipitates both the homologous and the heterologous C-test antigen; however, it does not react with test antigens in which M or Ge is present.

The cross-precipitin reactions of these four disaccharide antisera

 $<sup>\</sup>pm$  = faint turbidity when read with artificial illumination against dark background.

 $<sup>0 = \</sup>text{no precipitation}$ .

<sup>\*</sup> The immune sera were in all instances used in constant amounts of 0.2 cc. A dilution of serum in the proportion of two parts of serum to three parts of salt solution was prepared, and 0.5 cc. of this dilution, containing 0.2 cc. of the original serum, was added to 0.5 cc. of the varying dilutions of test antigens, in Tables I to IV.

<sup>†</sup> Test antigens were prepared by combining the respective glycosides with the protein of chicken serum.

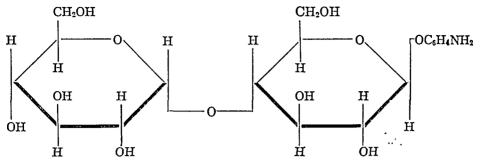
appear at first difficult of interpretation, but further consideration of the stereochemical structures of the four glycosides affords an insight into the complexities of the cross-reactions. Each of the disaccharide glycosides has in common a large chemical grouping; namely, a glucose molecule substituted by the aglucon—C<sub>6</sub>H<sub>4</sub>N=N— combined in glucosidic union with the reducing group of the hexose. In the case of the gentiobioside and cellobioside, each glycoside contains a terminal

TABLE II

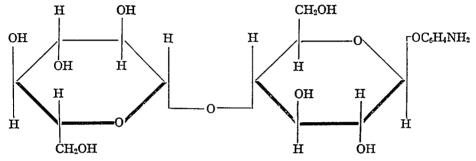
Heterologous Precipitin Reactions of Disaccharide Antiscra

Antisera prepared by immunization with:	Test antigens	Final	lilution of test an	tigens
immunization with:		1:5,000	1:10,000	1:20,000
C-Globulin	C Chicken serum M " " Ge " " L " "	++++ ± ++± ±	++++ ± ++ ±	+++± ± +± ±
M-Globulin	C Chicken serum M " " Ge " " L " "	+++ ++++ ++± ±	++± ++++ +± ±	+± +++± +
Ge-Globulin	C Chicken serum M " " Ge " " L " "	+++ ± ++++ ±	+++ ± +++± ±	++ ± ++± ±
L-Globulin	C Chicken serum M " " Ge " " L " "	++± ± ± +++±	++± ± ± +++	++ ± ± +++

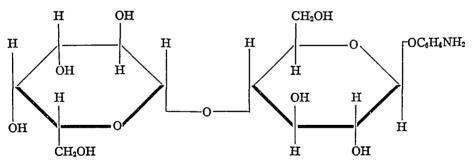
 $\beta$ -glucose molecule substituted in positions 6 and 4 respectively of the glucose molecule bearing the aglucon. The maltoside, on the other hand, has an  $\alpha$ -glucose molecule substituted in position 4, whereas the lactoside has a  $\beta$ -galactose molecule in this same position. The constitutional and configurational relationships of these four glycosides may best be understood by the following graphic formulae:



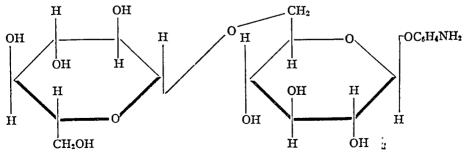
p-Aminophenol  $\beta$ -maltoside



p-Aminophenol  $\beta$ -cellobioside



p-Aminophenol  $\beta$ -lactoside



p-Aminophenol  $\beta$ -gentiobioside

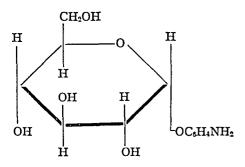
From the graphic formulae it can be seen that the carbohydrate radicals of the cellobioside and gentiobioside are each composed of two units of glucose combined in glucosidic union. In the case of the cellobioside, the union of hexose molecules is on the fourth carbon atom of the glucose molecule bearing the aglucon; in the gentiobioside this union is on carbon atom 6. Apparently as a result of the similarity in structure, and because of the  $\beta$  configuration of the terminal glucose molecule common to both glycosides, antigens containing either C or Ge show reciprocal cross-reactions in the corresponding antisera. The position of linkage of hexose to hexose is apparently less important in determining specificity than is the configuration of the terminal hexose. The configuration of the individual carbon atoms of the terminal hexose is also an important factor in determining specificity, since an antigen containing L does not react in C antiserum despite the fact that the structures of C and L are identical save in respect to the spatial arrangement of the H and OH groups on the fourth carbon atom of the terminal hexose.

Although the M-test antigen fails to precipitate either in C- or Geantiserum, it is a striking fact that both C- and Ge-test antigens precipitate in an antiserum prepared by immunization with maltoside-globulin. When one considers these cross-reactions with reference to the chemical structure of the three glycosides, it appears that the specificity of the immunological response is more sharply defined when the terminal hexose in the antigenic molecule has the  $\beta$  rather than the  $\alpha$  configuration. The reason for this is not known.

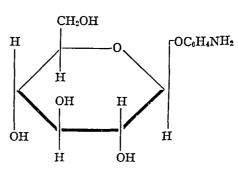
It has been previously pointed out that the four glycosides, M, C, Ge, and L, each possess in common a large molecular grouping; namely, a glucose molecule bearing the aglucon—C<sub>t</sub>H<sub>4</sub>—N=N—. In view of this fact one might expect the corresponding antisera to show marked cross-reactions with test antigens containing any one of the heterologous glycosides. That this is not the case, however, is evident from the results of the precipitin tests given in Table II. It may be concluded, therefore, that the large grouping common to all the disaccharide antigens exerts but little influence on serological crossing and that in those instances in which this phenomenon occurs the reactions are determined primarily by similarities in the configuration of the terminal hexose of the glycosides.

One striking exception, however, has been encountered in the case of the lactoside, in which the terminal hexose is  $\beta$ -galactose. An antiserum to the lactoside would, on the basis of the preceding hypothesis, not be expected to precipitate an antigen having a terminal  $\beta$ -glucose molecule. However, it has been shown that L-antiserum contains precipitins reactive with C-test antigen, although the reaction is not reciprocal. There is at present no adequate explanation for this phenomenon. It may be pointed out that Landsteiner and Lampl (4) encountered a series of non-reciprocal reactions in the course of investigations on the specificity of antisera to o-aminobenzene sulfonic acid, and o-aminobenzoic acid. Similarly Heidelberger and Kendall (5) have described in detail an analogous series of non-reciprocal precipitin reactions employing antisera prepared by immunization of rabbits with R-salt-azo-benzidine-azo-crystalline egg albumin and native crystalline egg albumin.

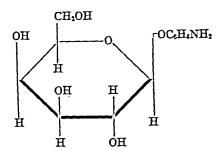
3. Heterologous Precipitin Reactions of Disaccharide Antigens in Monosaccharide Antisera.—In order to gain further insight into the factors which determine the serological specificity of disaccharide antigens, the latter were tested in the immune sera prepared by the immunization of rabbits with antigens containing the azophenol glycosides of  $\alpha$ - and  $\beta$ -glucose and  $\beta$ -galactose combined with horse serum globulin. The structural relationship of the hexosides from which the corresponding monosaccharide antigens were prepared is represented by the following graphic formulae:



p-Aminophenol  $\alpha$ -glucoside



p-Aminophenol β-glucoside



p-Aminophenol β-galactoside

From the above formulae it can be seen that the  $\alpha$ - and  $\beta$ -glucosides differ from one another in the stereochemical arrangement of the carbon atom bearing the aglucon. The remaining five carbon atoms of each hexoside have the same stereochemical pattern. The  $\beta$ -galactoside differs from the  $\beta$ -glucoside in that in each instance the H and OH groups on the fourth carbon atom are interchanged. Before turning to the results of the precipitin tests of the disaccharide test antigens in the monosaccharide antisera, it must be recalled that the terminal hexose of both the cellobioside and the gentiobioside is  $\beta$ -glucose, while the terminal molecule of the maltoside is  $\alpha$ -glucose, and of the lactoside,  $\beta$ -galactose.

From the results of the precipitin reactions of disaccharide antigens in monosaccharide antisera (Table III) it is interesting to observe that only the test antigen containing the maltoside, the terminal hexose of which is  $\alpha$ -glucose, reacts in  $\alpha$ -antiserum. The two test antigens containing the cellobioside and gentiobioside, both of which have terminal  $\beta$ -glucose radicals, react only in  $\beta$ -antiserum, and finally the lactoside test antigen, the terminal hexose radical of which is galactose, reacts only in  $\beta$ -galactoside antiserum. The results of these serological tests support the view that the stereochemical configuration of the terminal hexose in disaccharide antigens is the dominant factor in determining their serological crossing in monosaccharide antisera.

4. Precipitin Reactions of Monosaccharide Antigens in Disaccharide Antisera.—In order to determine whether the monosaccharide test antigens react with the disaccharide antisera, the latter were tested for the presence of precipitins reactive with  $\alpha$ -,  $\beta$ - and Ga-test antigens. The results of the precipitin tests are given in Table IV. From

the results given in Table IV it may be seen that only the  $\alpha$ -test antigen reacts in M-antiserum, whereas the  $\beta$ -test antigen reacts in both

TABLE III

Precipitin Reactions of Disaccharide Antigens in Monosaccharide Antisera

Antisera prepared by immunization with:	т	'est anti	gens	Final	dilution of test a	ntigens
immunization with:				1:5,000	1:10,000	1:20,000
lpha-Globulin	α C C M L Ge	Chicker " " "	serum "  "  "	+++ ± ++± 0	++± 0 ++± 0 0	++± 0 ++ 0 0
β-Globulin	β C C M L Ge	Chicken "  "  "  "	serum " " "	++++ +++ ± 0 +++	++++ ++± 0 0 +++	+++± ++ 0 0 ++
Ga-Globulin	Ga C C M L Ge	Chicken " " "	u serum u u u u	+++ 0 0 +± 0	++± 0 0 ++ 0	++ 0 0 ++ 0

TABLE IV

Precipitin Reactions of Monosaccharide Antigens in Disaccharide Antisera

		l dilution n serum a		Fina chicke	l dilution n serum t	of β antigen	Final dilution of Ga chicken serum antigen			
Antisera prepared by immunization with:	1:5,000	1:10,000	1:20,000	1:5,000	1:10,000	1:20,000	1:5,000	1:10,000	1:20,000	
C-Globulin	± +++ 0 ±	± +++ 0 ±	± ++ 0 ±	++± +++ 0 ++±	++ ++± 0 ++±	++ ++ 0 ++	0 0 ++ 0	0 0 +± 0	0 0 + 0	

C- and in Ge-antiserum as well as in M-antiserum. It has been previously shown that both C- and Ge-test antigens, the carbohydrate radicals of which both contain a terminal  $\beta$ -glucose molecule, react in

M-antiserum. It is not surprising therefore, that an antigen containing a monosaccharide radical having the  $\beta$ -glucose configuration also reacts in the same serum. It is also evident from Table IV that the Ga-test antigen, which possesses a terminal  $\beta$ -galactose molecule, reacts only in L-antiserum. These serological findings again emphasize the fact that the configuration of the terminal hexose is a dominant factor in determining the specificity of antibodies induced by disaccharide antigens.

TABLE V

Inhibition of Precipitins in Cellobioside-Globulin Antiserum by Homologous and Heterologous Glycosides\*

Inhibiting glycoside	Test a	ntigens (final dilution 1:5,	(000)
Indicating gry costace	c	Ge	β
None	++++	++	++±
α	++++	十土	++
β	++++	0	0
Ga	++++	++	++
c	0	0	0
M	+++±	+=	++
L	+++±	+±	++±
Ge	+++±	0	o o

<sup>\*</sup> The technique of the specific inhibition tests given in Tables V to VIII was as follows: 0.3 cc. of a 0.1 M solution of the different p-aminophenol glycosides was added to 0.2 cc. samples of immune serum. The mixtures were incubated for 2 hours at  $37^{\circ}$ C. and to them was then added 0.5 cc. of a 1:2,500 dilution of the reactive test antigens. The tubes were again incubated for 2 hours at  $37^{\circ}$ C. and the final readings were recorded after the tubes had stood overnight in the ice box.

# II. Specific Inhibition Tests

1. Cellobioside-Globulin Antiserum.—The selective specificity of the precipitins in cellobioside-globulin antiserum for test antigens containing the cellobioside, the gentiobioside, and the  $\beta$ -glucoside can be seen from the results of the specific inhibition tests given in Table V.

An analysis of the data presented in Table V shows that the specific reaction between C-antiserum and its homologous test antigen can be inhibited only by C, whereas the cross-reaction of Ge-test antigen in C-antiserum is inhibited by C, Ge, and by  $\beta$ . These three glycosides

each have a terminal  $\beta$ -glucose radical. It must be pointed out that the heterologous reactions, though somewhat diminished, are not completely inhibited by  $\alpha$  or by M, both of which are glycosides having a terminal  $\alpha$ -glucose radical. Likewise the cross-reaction between C-antiserum and Ge-test antigen is not inhibited by L or by Ga, both of which contain a terminal  $\beta$ -galactose radical. Similarly, the cross-reaction between  $\beta$ -test antigen and C-antiserum is inhibited by C, Ge, and by  $\beta$ , but by none of the other glycosides.

The results of the specific inhibition tests emphasize the fact that an antigen containing a cellobiose radical gives rise to antibodies which are specific for this particular carbohydrate grouping. Since the reac-

TABLE VI
Inhibition of Precipitins in Gentiobioside-Globulin Antiscrum by Homologous and
Heterologous Glycosides

Inhibiting glycoside	Test	antigens (final dilution 1:5,	000)
Inflorting glycoside	Ge	С	β
None	+++	十土	++±
α	++±	+±	++
β	++±	0	0
Ga	++±	+±	++
С	++±	0	0
M	++±	十士	++
L	++±	十士	++
Ge	0	0	o o

tion between C-antigen and homologous antiserum cannot be inhibited by the glycoside of gentiobiose, a disaccharide which is isomeric with cellobiose, it may be concluded that the position of the biose linkage is important in determining the biological specificity of disaccharide antigens. From the fact that the heterologous reactions of  $\beta$ - and Ge-test antigens in C-antiserum can be inhibited only by glycosides containing a terminal  $\beta$ -glucose molecule, it may be further concluded that the specificity of the serological cross-reactions is determined primarily by the configuration of the terminal hexose residue of the cellobioside.

2. Gentiobioside-Globulin Antiserum.—The results of the specific inhibition of precipitins in gentiobioside-globulin antiserum by the

homologous and heterologous glycosides are summarized in Table VI. From the results presented in Table VI it can be seen that only the gentiobioside inhibits the reaction between Ge-antiserum and homologous test antigen. The fact that the homologous reaction cannot be inhibited by the cellobioside, a derivative which is identical with the gentiobioside in structure except for the position of the biose junction, further substantiates the importance of the position of intermolecular linkage in determining the specificity of disaccharide antigens. As in the case of the cellobioside antiserum, the heterologous precipitin reactions of Ge-antiserum are in each instance inhibited by glycosides

TABLE VII

Inhibition of Precipitins in Maltoside-Globulin Antiserum by Homologous and Heterologous Glycosides

Inhibiting gly- coside		Test antig	ens (final dilution f	1:5,000)	
coside	м	α	β	С	Ge
None	++++	++±	+++	++±	++±
α	+++±	0	0	0	0
β	+++±	++±	0	0	0
Ga	+++±	++±	+++	++	++
c	+++±	++±	±	0	0
М	0	0	0	0	0
L	+++±	++±	+++	++	+±
Ge	+++±	++±	0	0	0

containing a terminal  $\beta$ -glucose molecule, and not by those having an  $\alpha$ -glucose or  $\beta$ -galactose configuration.

3. Maltoside-Globulin Antiserum.—It will be recalled that an antiserum prepared by immunization with an antigen containing azophenol maltoside cross-reacts with test antigens in which the terminal hexose residue is either  $\alpha$ - or  $\beta$ -glucose, but does not react with antigens in which the terminal hexose is  $\beta$ -galactose. The results of the specific inhibition of precipitins in M-antiserum by the different glycosides are given in Table VII. From the results of the specific inhibition tests given in Table VII it can again be seen that precipitation of M-test antigen in homologous antiserum is inhibited only by the maltoside. The failure of the other glycosides to inhibit the homologous

gous precipitin reaction indicates that the maltoside antigen gives rise to immune bodies specific for the disaccharide radical in question.

It has previously been pointed out that the terminal hexose of maltose is  $\alpha$ -glucose. The configurational relationship of the six carbon atoms of this portion of the maltoside is in all respects identical with that of the simple monosaccharide derivative,  $\alpha$ -glucoside. On the basis of this configurational identity, and in view of the fact that the specificity of the cross-precipitins elicited by antigens containing a disaccharide radical is determined primarily by the terminal hexose, the precipitation of azophenol  $\alpha$ -glucoside in M-antiserum may be considered as approaching an homologous reaction. It is therefore to be anticipated that this reaction would be inhibited only by those glycosides possessing an  $\alpha$ -glucose configuration. That this is indeed the case may be seen by referring to Table VII, the results of which show clearly that neither B, Ge, nor C inhibit the reaction of  $\alpha$ -test antigen in M-antiserum. It may be concluded that the configuration of the terminal hexose of the M-immunizing antigen so orients the specificity of the antibodies induced that the latter, as evidenced by the inhibition tests, remain predominantly specific for the  $\alpha$ -glucose configuration.

The cross-reactions of M-antiserum with the heterologous test antigens  $\beta$ , C, and Ge can be understood by comparing the similarity in chemical constitution of the three glycosides with that of the homologous maltoside. In each glycoside the terminal hexose is glucose. the maltoside this hexose has the  $\alpha$ -configuration, whereas in the  $\beta$ glucoside, the cellobioside, and the gentiobioside, the terminal hexose is  $\beta$ -glucose. In all of these glycosides the five end carbon atoms are identical in stereochemical structure. This identity in structure may explain the cross-reactions of test antigens containing the terminal  $\beta$ -glucose configuration in M-antiserum. Similarly, it might be expected that test-antigens containing a terminal α-glucose molecule would reciprocally react in the antisera prepared by immunization with antigens containing a terminal  $\beta$ -glucose configuration. already been pointed out, however (cf. Table II), that in general this is not the case, although certain C- and Ge-antisera have been obtained which show a slight degree of crossing with both  $\alpha$ - and M-test antigens, when the latter are used in high concentration. It appears,

therefore, that disaccharide antigens containing a terminal  $\beta$ -glucose molecule give rise to antibodies which in general cross-react with test-antigens having a  $\beta$ -glucose molecule and not with those in which the terminal hexose has the  $\alpha$  configuration. This fact appears to be true only for antigens containing the azophenol glycosides of the  $\beta$ -disaccharides and not for corresponding glucoside of the monosaccharide,  $\beta$ -glucose. The reason underlying this unusual phenomenon cannot as yet be defined with certainty. By reference to molecular models of the two disaccharides, maltose and cellobiose, it is at once apparent that the spatial relationship of the polar groups of the two hexoses is in each instance different. This difference in the orientation of these groups may modify, or even mask, the influence which the individual polar groups might otherwise exert upon the specificity of disaccharide antigen containing two hexose molecules.

It has previously been pointed out that the cross-reactions of  $\beta$ -, C-, and Ge-test antigens in M-antiserum may be attributed to the identity in configuration of the five end carbon atoms of each of these glycosides. It might be expected, therefore, that any glycoside having this common configuration would inhibit the reaction of  $\beta$ -, C-, or Ge-test antigens in M-antiserum. Indeed, this has proved to be the case, for it may be seen from Table VII that the cross-reactions of the maltoside-globulin antiserum with any one of the heterologous test antigens containing a terminal  $\beta$ -glucose molecule are inhibited by any one of the  $\beta$ -aminophenol glycosides, in which the terminal hexose molecule is glucose, irrespective of its configuration. However, when the configuration of one of the five terminal polar groups is altered, as in the case of the galactoside or lactoside, the latter glycosides fail to inhibit the cross-reactions between M-antiserum and test antigens containing a terminal glucose molecule.

4. Lactoside-Globulin Antiscrum.—The selective specificity of the antibodies in lactoside antiscrum may be seen from the results of the inhibition tests given in Table VIII.

From data presented in Table VIII, it is seen that the antigen containing the lactoside gives rise to immune bodies which are specific, since the precipitation of L-test antigen in homologous antiserum can be inhibited only by the homologous glycoside. The cross-precipitation of Ga-test antigen in L-antiserum is likewise inhibited only by

the glycoside of lactose and galactose. This observation once more confirms the view that the terminal hexose of disaccharide antigens exerts a dominant influence in determining serological crossing. On the basis of this concept, it would be expected that L-antiserum would react only with test antigens containing L and Ga. However, it has already been seen (Table II) that C-test antigen reacts in L-antiserum despite the fact that the terminal hexose of the cellobioside is glucose, while that of the lactoside is galactose. The only portion of the cellobioside and lactoside which is identical in configuration is therefore the glucose molecule bearing the aglucon. The serological activity of C-test antigen in L-antiserum might be ascribed to this common group-

TABLE VIII
Inhibition of Precipitins in Lactoside-Globulin Antiscrum by Homologous and
Heterologous Glycosides

Inhibiting glycoside	Test	antigens (final dilution 1:5	,000)
Thin brend glycoside	L	Ga	С
None	+++	+±	++
α	+++	+±	十土
β	+++	+±	+±
Ga	+++	0	+±
С	+++	十士	0
M	+++	十士	十士
L	0	0	0
Ge	+++	+±	+±

ing. It must be remembered, however, that this grouping is also common to the maltoside and gentiobioside, yet antigens containing the latter glycosides fail to react in L-antiserum. Moreover the two glycosides, Ge and M, do not inhibit the cross-reaction of C-test antigen in L-antiserum. At present there is no adequate explanation for this unusual cross-precipitin reaction, nor do the results of the specific inhibition tests throw any light on the situation. The question must await further investigation before a definite explanation can be given.

### DISCUSSION

In attempting to understand the intricate serological relationships between antigens containing the four disaccharides, gentiobiose, cellobiose, lactose, and maltose, it is necessary to have clearly in mind a picture of the stereochemical structure of each disaccharide. The reducing disaccharides, maltose, gentiobiose, lactose, and cellobiose, are compounds of two hexose molecules joined in glycosidic union through the reducing group of one of the monosaccharide constituents. The point of attachment of one hexose to the other, and the nature of the linkage (i.e. whether the terminal hexose has an  $\alpha$  or  $\beta$  configuration) have been carefully ascertained in the case of the four disaccharides in question, and as a result their chemical structures are fully comprehended. With an exact knowledge of their structures, the disaccharides become excellent substances for study in correlating changes in chemical constitution with differences in biological specificity.

The conversion of these four disaccharides into their corresponding p-aminophenol glycosides (3) is without doubt accompanied by no change in the lactal ring structure of the hexose units. The glycosides may be regarded as built up from two units of hexoses in pyranoid form, with the remaining reducing group replaced by the aglucon  $-C_tH_4NH_2$ . The three glycosides of maltose, cellobiose, and lactose, have a common structure and are distinguishable only in that each has a different configuration. The gentiobioside, on the other hand, differs structurally from its isomers in that the point of attachment of the terminal  $\beta$ -glucose molecule is on the sixth carbon atom of the glucose molecule bearing the aglucon. By referring to the graphic model formulae these structural and configurational relationships may be clearly seen.

From the results of the specific inhibition tests and the heterologous precipitin reactions it may be seen that these structural and configurational relationships have a distinct and definite influence in determining the immunological specificity of antigens containing these disaccharides. The present study emphasizes the fact that the specificity of the serological cross-reactions of disaccharide antigens is determined by the configuration of the terminal hexose molecule. In a study of the immunological properties of peptides, Landsteiner and van der Scheer (6) have shown that the specificity of antigens containing amino benzoylated peptides depends primarily upon the structure of the terminal amino acid, and to a less degree upon the other amino acids in the peptide chain.

Although each of the disaccharide antigens contains in common an azophenol  $\beta$ -glucoside radical, this second hexoside grouping does not appear to be of importance in determining serological crossing; however, its presence does confer upon each antigen certain specific properties. The rôle of this common hexose molecule bearing the aglucon is demonstrated by the fact that the homologous reactions between disaccharide test antigens and the corresponding antisera are not inhibited by simple hexosides conforming in configuration and structure to the terminal hexose molecule of the disaccharide. It must be borne in mind, therefore, that the individual specificity of disaccharide antigens is dependent upon the molecular pattern of the glycoside Thus it has been shown that antigens containing radical as a whole. glycosides as closely related as are the gentiobioside and cellobioside, may be specifically and sharply differentiated serologically by means of inhibition reactions.

From a comparison of the immunological properties of the different disaccharide antigens, it is evident that when the terminal hexose has the  $\beta$  configuration the specificity of the antibody response is more sharply defined than when this configuration is of the  $\alpha$  type, for it has been shown that although C- and Ge-test antigens react in M-antiserum, the reverse does not hold true.

A striking analogy to the lack of reciprocal cross-reactions is found in the case of the capsular polysaccharides of Pneumococcus Types III and VIII. It has been observed that the specific polysaccharide of Type VIII Pneumococcus reacts both in Type VIII and Type III antipneumococcus serum, whereas the Type III carbohydrate reacts only in the homologous antiserum.

## SUMMARY

The results of the present study indicate that by means of serological reactions it is possible to differentiate selectively the p-aminophenol glycosides of maltose, cellobiose, gentiobiose, and lactose. The immunological specificity of disaccharide-protein antigens prepared from these derivatives, irrespective of the nature of the conjoined protein, is determined by (1) the glycoside molecule as a whole, (2) the configuration of the terminal hexose molecule, and (3) the position of linkage of the two hexose units in the carbohydrate radical. The specific-

ity of the antibodies induced by the disaccharide antigens appears to be more sharply defined when the configuration of the terminal hexose is of the  $\beta$  rather than of the  $\alpha$  type.

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# STUDIES UPON MINUTE HEMOLYTIC STREPTOCOCCI

# I. THE ISOLATION AND CULTURAL CHARACTERISTICS OF MINUTE BETA HEMOLYTIC STREPTOCOCCI\*

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In a recent brief report (1) we discussed the isolation of small, amphophilic cocci which possess the power of producing the beta type of hemolysis in poured plates of rabbit's blood, sugar-free agar. These minute organisms resembled the ordinary type of beta hemolytic streptococci in many of their morphological and cultural characteristics. They were isolated from the rhinopharynx in a variety of diseases and from normal human beings, but were recovered most frequently from the throats of individuals suffering from glomerular nephritis or progressive rheumatic infection. These organisms occur in pairs, short chains and masses and are one-half to two-thirds the size of ordinary beta hemolytic streptococci. While it seems unlikely that these organisms have not been previously observed, a thorough search of the available literature has failed to reveal any description of them. In this report their cultural characteristics will be described.

### Methods

Technique of Primary Isolation.—The majority of our strains have been isolated from the throats of human beings and we believe that the method of swabbing the throat is of prime importance. We have found that cotton-tipped sterile twisted wire swabs are ideal for this purpose. They must not be tipped with too much cotton. The ordinary cotton-tipped, wooden applicators which one generally encounters on hospital wards are too bulky and too difficult to maneuver in the process of swabbing a throat. It is our practice to rub thoroughly each tonsil or tonsillar fossa, exploring the tonsillar crypts, if any are present, and

<sup>\*</sup> Read in abstract before the 35th Annual Meeting of the Society of American Bacteriologists, Philadelphia, December 29, 1933.

then to swab the tissues behind the pillars and on the posterior pharyngeal wall. Two of the swabs held together are used in this procedure.

The swabs are transferred to small test tubes containing 1 cc. of sterile physiological saline and are vigorously shaken in the salt solution. One 4 mm. loopful of this suspension is inoculated into a tube containing 12 cc. of melted 2 per cent, sugar-free, beef infusion agar (temperature 45°C.), 1 cc. of sterile defibrinated rabbit's blood is added and the whole is thoroughly mixed and poured into a sterile Petri dish. The following formula is used in preparing the agar. 1 pound of freshly ground, lean, raw beef is added 1 liter of tap water and the mixture is infused overnight in the ice box. On the following day the fat is removed and the residue is heated to between 85-90°C. for 30 minutes and is filtered through grey French filter paper. To the filtered broth 5 gm. of sodium chloride and 10 gm. of neopeptone per liter are added and the whole is brought to a temperature of 95°C. The pH is adjusted to 7.8 by adding twice normal NaOH. The broth is autoclaved at 15 pounds pressure for 10 minutes and is immediately filtered through grey French filter paper to remove the precipitate. 20 gm. of bacto-agar are added to each liter of broth and the mixture is autoclaved for 15 minutes under 15 pounds pressure. The agar is then filtered to remove the resulting precipitate and is poured in 12 cc. amounts into sterile tubes and these in turn are sterilized for 10 minutes under 15 pounds pressure. The final pH of the agar prepared in this fashion ranges from 7.4 to 7.6. think that it is essential to use neopeptone in the preparation of the medium because in our experience ordinary peptones seem to inhibit the growth of the minute hemolytic streptococci.

The inoculated plates are incubated at 37°C. for 24 hours before being examined for the minute hemolytic areas which denote the presence of the organisms. In order to distinguish these areas, a good source of light is required, and on dark days we have found that the light from a substage microscope lamp serves very well as a source of illumination. If no areas of hemolysis are visible the plates are reincubated, with daily examinations, for an additional period of 72 hours before finally being discarded. If, however, suspicious areas of hemolysis are seen, they are examined for the presence of a colony under the low power of an ordinary microscope. Final identification of the colony is accomplished by fishing and transferring it to sugar-free beef infusion rabbit's blood broth in which, after 24 to 48 hours incubation at 37°C., growth turbidity and hemolysis will be evident if the organism is a minute hemolytic streptococcus. In fishing we have found that it is necessary to use a colonial microscope as otherwise mixed cultures will result.

Colonial Morphology, Hemolysis and Growth in Blood Agar.—In studying these characteristics the organisms were grown in sugar-free, 10 per cent, rabbit's blood agar poured plates at a temperature of 37°C. After 18, 24 and 48 hours, the size and morphology of the colony and the approximate diameter of the area of hemolysis were determined by studying colonies under the lower power of an

ordinary microscope. A standardized ocular micrometer was used in making the measurements.

Fermentation Reactions.—10 per cent sterile lactose, mannitol, salicin, trehalose and sorbitol solutions were added to tubes of sugar-free beef infusion broth in amounts sufficient to make the final concentration of these substances in the broth 0.5 per cent. The five sugar media were each inoculated with 0.1 cc. of an actively growing strain and were incubated for 4 days at 37°C. If an abundant growth occurred the presence or absence of the production of acid was determined by adding Andrade's indicator.

Reduction of Methylene Blue Milk.—R. C. Avery's (2) method of preparing the methylene blue milk medium was used. This medium with plain milk controls was inoculated by adding 0.1 cc. of an actively growing culture to 5 cc. of each medium. The cultures were incubated at 37°C. for 1 week and were examined each day for evidence of the reduction of methylene blue.

Hydrolysis of Sodium Hippurate.—The method described by Ayers and Rupp (3) was employed. The cultures were incubated at 37°C. for 4 days. Known positive cultures and uninoculated control tubes were included in each series.

Fibrinolytic Activity.—Dr. W. S. Tillett has tested a number of our strains for their fibrinolytic activity, using the method recently described by Tillett and Garner (4).

Final pH.—The final pH attained by the various strains of minute hemolytic streptococci after 3 days of growth in 1 per cent dextrose broth was determined by the methods of O. T. Avery and Cullen (5). Readings were made upon the supernatant broth of centrifuged cultures. Brom-cresyl green was the indicator used and the readings were checked against standards of brom-cresyl green phthalate-NaOH buffer solutions. All of our strains have been checked two or more times and where a difference of more than 0.2 pH was obtained the results were averaged. Because of the natural error in these determinations the results are expressed in terms of ±0.1 pH.

#### RESULTS

Since the time of the initial isolation of the organisms from the throat of an individual ill with latent glomerular nephritis we have isolated and observed over 200 strains of minute beta hemolytic streptococci. These have been obtained from individuals ill with glomerular nephritis, rheumatic infection, other acute or chronic diseases and from normal human beings. Upon two occasions they have been recovered in pure culture from pus removed from acutely inflamed paranasal sinuses and in one instance from the pus from an abscess of the deep tissues of the chest.

In the primary isolation in rabbit's blood agar poured plates the

small areas of hemolysis are not usually evident until after 24 hours of incubation at 37°C. It is important that the blood agar plates be free from sediment and from bits of unmelted agar because these may easily be confused with true hemolysis. When the areas of hemolysis first become visible the colony cannot be distinguished by means of the unaided eye, and resort must be had to the use of the low power of an ordinary microscope. At this stage of development, the colony appears as a small, finely granular, roughly circular object ranging in size from 18 to 30 microns and surrounded by a relatively large area of true beta type hemolysis. Occasionally the colonies appear to be wrinkled and crenated or they may have a curious tetradic appearance. Rarely have the colonies been oval in the primary culture. By the end of 48 hours incubation they are visible to the naked eye, although, in certain instances, 96 hours of incubation were required before they were visible. In the first stages of development the ratio of the diameter of the area of the hemolysis to the diameter of the colony is roughly from 4 to 1, to 10 to 1. With further incubation this ratio decreases so that by the end of 48 hours the ratio is generally 3 or 4 to 1.

The growth from the first transfer of a colony to sugar-free rabbit's blood broth may be very slow. It is therefore advisable to incubate these cultures for at least 48 hours if no growth is visible at the end of The second transfer should also be made in a blood broth medium in order that the strain may become well established. films made from cultures in liquid medium and stained by Gram's method the organisms appear as minute cocci occurring singly, in pairs, in short chains and in small and large masses. coccus is one-half to two-thirds the size of the ordinary beta hemolytic streptococcus and it stains indifferently with Gram, some strains being The individual strongly Gram-positive while others are Gram-negative.

The organisms grow sparsely in plain sugar-free beef infusion broth and are maintained with difficulty in such a medium. In 0.1 per cent dextrose beef infusion broth growth is generally diffuse and abundant in the first two or three subplants from blood broth but unless care is exercised the organisms soon tend to die. Sugar-free blood broth appears to be a good medium for carrying strains of them.

If one inoculates a loopful of these organisms upon the surface of

a sugar-free, 5 per cent, rabbit's blood agar plate and spreads the inoculum with a glass rod, a fine mist of colonies will be observed after 18 to 24 hours of incubation. Hemolysis may or may not be visible at this time. However, at the end of 48 hours incubation marked hemolysis is present. When well spaced the colonies have the following characteristics. They may be dewdrop, flat peaked or peaked in outline. They are generally smooth and shiny although strains showing glazed, granular or wrinkled surfaces have been encountered. The hemolyzed agar about the colony is frequently depressed and pitted and the colonies themselves are dry and tenacious, and are removed with difficulty from the surface. An incubation period of from 5 to 6 days is required before the colonies reach their maximum size.

These streptococci are facultative anaerobes and can be grown on blood agar in anaerobic jars or in Noguchi's medium. They are difficult to maintain in stock cultures because they die out rather rapidly in ordinary media when left either at room temperature or at a temperature of  $\pm 4^{\circ}$ C. An easy method of preserving them is to inoculate Noguchi tubes containing 10 cc. of rabbit's blood broth with 0.1 cc. of culture and, after 24 hours of incubation at 37°C., to seal the tubes with sterile vaseline and to store them at  $\pm 4^{\circ}$ C. Cultures may be maintained for at least 4 months under these conditions.

In passing we must state that in the course of their primary isolation in rabbit's blood agar plates we have noted other microorganisms which tend to produce very small colonies and small zones of hemolysis. Among these are small forms of *Hemophilus hemolyticus*, members of the *Neisseria* group, minute *alpha* hemolytic streptococci and a hemolytic Gram-positive coccus which is as yet unidentified. All of these forms may be confused with minute streptococci as far as their gross appearance in blood agar plates is concerned.

The minute streptococci we have studied tend to fall into two main groups, the first consisting of strains which ferment salicin and tre-halose, and the second those which possess the *Streptococcus pyogenes* type of fermentation. Several strains showing irregular powers of fermentation were placed in a third group. We feel, however, that these strains represent examples of other groups which as yet have been encountered but rarely and we do not consider that these organ-

TABLE I
Cultural and Biochemical Characteristics of Group I Minute Hemolytic Streptococci

		Growth	in blood	l agar		Fern	nentat	ion of		thylene	sodium	per cent
Strain	Source	Cole Hemo	ony olysis in 1	nm.	S.G	nitol	п	lose	tol	Reduction of methylene blue	Hydrolysis of hippurate	Final pH in 1 per dextrose broth (# 0.1(pH)
		18 hr.	24 hr.	48 hr.	Lactose	Mannitol	Salicin	Trehalose	Sorbitol	Reduc	Hydre	Final dex
Hy. I	Normal	$0.108 \\ \hline 0.648$	0.198 0.990	0.378 1.22	_	_	+	+	_	-	-	4.9
Hy. II	**	$\frac{0.108}{0.630}$	$\frac{0.216}{1.01}$	0.360 1.26	-	_	+	+	_	-	-	4.9
Ve.	ll.		-	0.162 0.752	_	_	+	+	_		_	4.8
S		-	-	0.252 0.774	-	_	+	+	-	_	_	4.8
My.	66	$0.090 \over 0.324$	$\frac{0.126}{0.404}$		-	-	+	+	_	_		4.9
Wi.	u	-	-	0.198 0.540	-	-	+	+	-	_	-	4.8
Pa.	"	-	-	0.180 0.738	-	-	+	+		-	_	4.8
De.	66	-		0.126 0.900	_	-	+	+	-	-		5.0
St.	66	0.090 0.324	$\frac{0.126}{0.522}$		1	-	+	+	-	-	-	5.0
N	Nephritis	-		0.126 0.450	-	-	+	+	-	-	-	4.7
M.H.W.	66	$0.045 \\ \hline 0.234$	0.045 0.252		-		+	+	-	-	-	4.8
Ha.	"	$\frac{0.090}{0.450}$	0.180 0.900		-	-	+	+	-	-	-	4.9

TABLE I-Continued

					011111							
		Growth	in blood	agar		Ferm	entati	on of		thylene	sodium	per cent
Strain	Source	Colo Hemo	ny lysis in n	nm.	356	nitol	g	nlose	tol	Reduction of methylene blue	Hydrolysis of hippurate	Final pil in 1 pe dextrose broth (** 0.1 pil)
		18 hr.	24 hr.	48 hr.	Luctose	Mannitol	Sallein	Trehaloso	Sorbital	Redu	II Miles	Find Find Find Find Find Find Find Find
103 H	Nephritis	-	0.054 0.288		-	-	+	+	-	_	-	4.8
Me.	<b></b>	_	-	0.244 0.576	-	-	+	+	-	-	-	4.9
Но.	u	-	$\frac{0.054}{0.324}$	0.274 0.864		_	+	+	-	-	-	4:8
65 F	"	-	-	0.396 0.990		-	+	+	-	-	-	4.9
Kl. I	"	0.072 0.378			-		+	+	_	-	_	4.8
Kl. II	"	0.054 0.360	0.090 0.540	0.256 1.03	-	-	+	+	-	-	-	4.8
Me.	46	_	0.108 0.425	0.288 0.936	-	-	+	+	-	-	-	5.0
W	86	-	_	0.180 0.720		_	+	+	_	_	-	4:.7
Pai.	66	-	-	0.198 1.08	-	-	+	+	-	-	-	4.7
D	66	-	0.072	0.306	-	-	+	+		-	-	4.7
40 E	"	0.027 0.234	0.054 0.324	0.126 0.468	-	_	+	+		-	-	4.9
Wh.	Rheumatic fever	-	0.126 0.378	0.162 0.844	-	_	+	+	-	-	-	4.7
Ta.	ec 11	-	-	0.126 0.504	.1	_	+	÷	-	-		4.7

=			TABLE I—	Concluded
St	rain	Source	Growth in blood agar  Colony Hemolysis in mm.	Fermentation of lose the sodium lose the sodiu
An.		Acute tonsil- litis	0.090 0.144 0 270	_
Ba.	- 1	""	$0.4\overline{14}   \overline{0.720}   \overline{0.882}  $	- + + - - - 4.8
Ch.		" " 0	$ \begin{array}{c cccc}  & 0.054 & 0.270 \\ \hline 0.360 & 1.01 & - \\ \hline 0.108 & 0.162 & 0.270 & - \\ \hline 0.550 & 0.162 & 0.270 & - \\ \hline \end{array} $	-   -   +   +   -   -   -   4.8
Jo.	s	carlet fever 0.	0.084 0.900	
Fo. I	Sir	nusitis	360 0.521 1.04 0.126 0.198 _	-   +   +   -   -   -   4.8
Fo. II	Sin	(antrum) usitis ethmoid)	0.404 0.720	
38 E	Phle	bitis $0.05$		_   +   +   -   -   -   4.6
Wr.	Absco che	0.21	6 0.414 0.918 7 0.0360 270	-   +   +   -   -   -   4.9
95 E	Diabe	7 - 1 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2	$ \begin{array}{c ccccc} \hline 0.288 & 0.864 \\ \hline 0.036 & 0.144 \\ \hline 0.252 & 0.604 \\ \end{array} $	+   +   -   -   -   4.9   +   +   -   -   -   4.8
sms consti	tute	2 +		1             1.0

isms constitute a true group in the same sense as do the organisms in

The majority of the strains fall into the first group. It is apparent from Table I that these strains tend to produce rather small colonies and in some instances such a retarded type of hemolysis that neither colony nor hemolysis are visible at the end of 24 hours. At the end of 18 hours incubation, with certain strains, the ratio of the diameter of the zone of hemolysis to the diameter of the colony varied from

4 to 1, to 9 to 1. At the end of 48 hours of incubation this ratio was in most instances 3 or 4 to 1. Ordinary beta hemolytic streptococci from human sources have a ratio of 3 or 4 to 1 from the time the colonies are first visible and preserve this ratio throughout the period of incubation.

All of the strains of minute hemolytic streptococci in this group fermented salicin and trehalose while none fermented lactose, mannitol or sorbitol. On the basis of their reaction in lactose, mannitol and salicin, they would be classed as belonging to the Streptococcus equi group. However, Edwards (6) has recently shown that almost all hemolytic streptococci of human origin ferment trehalose and not sorbitol while the majority of hemolytic streptococci from animal sources ferment sorbitol and not trehalose. An exception to this latter finding exists in the hemolytic streptococci which are isolated from cases of strangles in horses, which ferment neither trehalose nor sorbitol but do attack salicin. Therefore, while the organisms in this first group resemble typical equine streptococci in their ability to ferment salicin, their power of fermenting trehalose definitely indicates that they cannot be classed as typical Streptococcus equi strains.

Lancefield (7) has demonstrated by means of a precipitin reaction that the strains of beta hemolytic streptococci which ferment trehalose can be divided into two groups when tested by this serological method. In her Group A almost all of the strains are of human origin while in Group B many of the strains are derived from bovine sources. However, the Group B strains hydrolyze sodium hippurate and produce more acid in 1 per cent dextrose broth than do the Group A strains. None of the minute organisms hydrolyzed sodium hippurate but they differed from Lancefield's Group A strains in acid production in 1 per cent dextrose broth. Many of our organisms produced more acid than the Group A strains but less acid than the Group B strains.

Strains of minute hemolytic streptococci possessing a Streptococcus pyogenes type of fermentation were placed in a second group. A marked variation in the ratio of the diameter of the zone of hemolysis to the diameter of the colony was noted among the members of this group. Certain strains behaved in this respect as did members of the first group while others maintained a high ratio throughout the period of incubation. All fermented lactose, salicin and trehalose.

None hydrolyzed sodium hippurate or reduced methylene blue milk. A moderate amount of acid was produced in 1 per cent dextrose broth by the members of this group.

TABLE II

Cultural and Biochemical Characteristics of Groups II and III Minute Hemolytic

Streptococci

		Growt	h in bloo	d agar		Ferr	nentat	ion of	3	thylene	sodium	er cent
Strain	Source	Colony Hemolysis in mm.			ခုဒ္ဓ	itol	<b>a</b>	lose	tol	Reduction of methylene blue	Hydrolysis of hippurate	Final pH in 1 per cent dextrose broth (± 0.1 pH)
		18 hr.	24 hr.	48 hr.	Lactose	Mannitol	Salicin	Trehalose	Sorbitol	Redu	Hydr	Final dex
				Group	II							
Te. I	Normal	$0.108 \over 0.954$		0.234 $2.52$	+	_	+	+	-	-	-	4.6
Te. II		0.108 0.990	$\frac{0.162}{1.67}$	$\frac{0.234}{2.34}$	+	-	+	+	_	-	-	4.5
Te. III		$\frac{0.108}{0.540}$	0.180 0.900		+	_	+	+	_	_	_	4.7
Ha. I	<b>66</b>	$\frac{0.108}{1.01}$	$\frac{0.180}{1.30}$	0.270 2.25	+	_	+	+	_	_		4.8
Ha. II	<b>66</b>	$\frac{0.108}{1.01}$	$\frac{0.162}{1.28}$	$\frac{0.198}{2.25}$	+	_	+	+	-	_	_	4.8
Ma.	<b>cc</b>	$\frac{0.126}{1.01}$	$\frac{0.180}{1.98}$	$\frac{0.352}{2.97}$	+	_	+	+	-	-	_	4.8
Ru.	<b>cc</b>	$0.090 \over 0.270$	$\frac{0.126}{0.720}$	$\frac{0.270}{0.846}$	+	-	+	+	-	-	-	4.9
Pe.	Scarlet fever	$\frac{0.072}{0.540}$	$\frac{0.108}{1.55}$	$\frac{0.180}{3.24}$	+	-	+	+	-	-	-	4.7
Ha.	Broncho- pneumonia	0.090 0.342	$\frac{0.144}{0.504}$	$\frac{0.342}{0.954}$	+	-	+	+	-	-	-	4.9
86 E	Tuberculous kidney	0.072 0.432	$\frac{0.180}{0.900}$	$\frac{0.396}{1.35}$	+	_	+	+	-	-	-	4.8

TABLE II-Concluded

									===			
		Growtl	in blood	l agar		Ferm	entati	on of		thylene	sodium	er cent
Strain	Source	Colony Hemolysis in mm.			SG	itol	u	lose	lo	Reduction of methylens blue	Hydrolysis of hippurate	Final piff in 1 p dextrose broth (14 0.1 pif)
		18 hr.	24 hr.	48 hr.	Lactose	Mannitol	Salicin	Trehalose	Sorbitol	Reduc	Hydro hipi	Fland See 3
				Group 1	ш_						_	
61 E	Nephritis	-	0.900 0.360	0.180 0.720		_		~	-	-	_	5.4
106 E	Nephritis	_	_	0.180 0.540		_	+	_	_	-	_	5.2
101 F	Rheumatic fever	-		0.234 $0.684$		_	_	_	_	_	_	5.4
45 E	££ €£	0.072 0.432			_	-	_	+	-	-	_	5.2
22 E	Acute phar- yngitis	_	0.126 0.360			-	+	-	-	-	-	4.9
84 E		-	-	0.378 1.11	-	-	+	-	-	-	-	5.2

The third group comprised strains which possess weak powers of fermenting the test sugars. Three of the members of this group had the typical Streptococcus equi type of fermentation reactions, and the other three resembled Streptococcus subacidus as far as their fermentation reactions in lactose, mannitol and salicin were concerned. None of the six strains hydrolyzed sodium hippurate or reduced methylene blue milk and with one exception only a small amount of acid was produced in 1 per cent dextrose broth by the members of this group.

A certain number of strains from the first two groups have been tested for the presence of the fibrinolytic substance recently described by Tillett and Garner (4) but as yet none of the tested strains has shown this substance. Inasmuch as Tillett (8) has demonstrated that 98.8 per cent of beta hemolytic streptococci from human sources possessed this substance while only 11 per cent of beta hemolytic strepto-

cocci of animal origin were capable of bringing about fibrinolysis, i is apparent that the lack of the fibrinolytic substance in the minute streptococci further differentiates them from ordinary hemolytic streptococci of human origin. Preliminary studies of the antigenic structure of these organisms have shown that, upon the basis of Lancefield's precipitin reaction, they fall into two groups, which are different from those previously described (7). This observation further separates the minute streptococci from ordinary beta hemolytic streptococci.

# DISCUSSION

It is evident from this study of the cultural and biochemical properties of these minute hemolytic organisms, that, while they resemble ordinary beta hemolytic streptococci in many respects, they cannot be classified under any previously described groups of these latter organisms.

The individual organisms are smaller than ordinary hemolytic streptococci, and the colonies are much more minute. Their fermentation reactions in the test sugars resemble those described for ordinary hemolytic streptococci of human origin except in three instances in which they conformed with those described for the organisms isolated from strangles in horses. None of the strains hydrolyzed sodium hippurate nor did they reduce methylene blue milk. The majority of the strains produced a fair amount of acid when grown in 1 per cent dextrose broth. None of the tested strains showed the presence of the fibrinolytic substance. Thus, while all of the strains possess certain of the characteristics of ordinary beta hemolytic streptococci none of them completely conform with known species of these organisms.

We realize that the term "minute" hemolytic streptococci is probably not the best one that could be applied to the organisms which we have studied but from a descriptive and differential point of view this term serves the purpose of pointing out a striking characteristic of this group. It is likely that they represent either new species of the beta hemolytic streptococcus or that they are hitherto undescribed variants of the ordinary bela hemolytic streptococci. At present we are unable to give a final opinion upon this point and can only state

that we have not observed a reversion of these minute organisms to the larger forms of *beta* hemolytic streptococci.

# CONCLUSIONS

The cultural and biochemical characteristics of a group of minute hitherto undescribed *beta* hemolytic streptococci from human sources have been recorded and upon the basis of these cultural reactions it is suggested that these organisms may represent new species of the genus streptococcus.

We wish to thank Drs. David and Beatrice C. Seegal for their kindness in securing for us the numbered cultures used in our investigations.

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# STUDIES UPON MINUTE HEMOLYTIC STREPTOCOCCI

II. THE DISTRIBUTION OF MINUTE HEMOLYTIC STREPTOCOCCI IN NORMAL AND DISEASED HUMAN BEINGS\*

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(Received for publication, July 20, 1934)

In an accompanying communication (1) we have described the primary isolation and the cultural characteristics of a hitherto undescribed group of minute beta hemolytic streptococci. In this report, we shall discuss the occurrence of these organisms and that of ordinary beta hemolytic streptococci in the throats of normal and diseased human beings.

# Methods

The technique of obtaining throat cultures and the methods of primary isolation already described (1) were followed in this study, save in the case of those cultures obtained from the throats of individuals ill with scarlet fever or septic sore throat. The throat swabs from these patients were shaken in 3 cc. of sterile physiological saline instead of the usual 1 cc.

The period of time covered by this study dates from March 29, 1933, until June 12, 1934, 80 per cent of the cultures being taken between October 1, 1933, and April 8, 1934. All of the cultures from normal individuals were obtained during the first 3 months of 1934, that is, during the period of the year in which the incidence of ordinary beta hemolytic streptococci is at a high level in Baltimore (2). With the exception of the group of patients ill with glomerular nephritis and a special group of 40 normal individuals, almost all of the cultures were single. Many of the individuals in the nephritic group were swabbed repeatedly at weekly intervals, and in order to have results from a similar group of normal subjects we swabbed 40 individuals once a week for a 12 week period.

Over 75 per cent of the throats were swabbed by one individual and when it was possible the cultural results were checked independently by two of us. Repeatedly we have obtained cultures containing the minute organisms when routine throat

<sup>\*</sup> Read by title before the 26th Annual Meeting of the American Society for Clinical Investigation, Atlantic City, April 30, 1934.

cultures failed to show them. In practically all instances the identity of the ordinary beta hemolytic streptococci and of the minute hemolytic streptococci was confirmed by the morphological appearance of the organisms and their production of hemolysin in sugar-free rabbit's blood broth.

Minute hemolytic streptococci were infrequently found in single cultures from the throats of normal individuals. As is shown in Table I, in a series of 404 cultures from a like number of normal

Incidence of Beta Hemolytic Streptococci and Minute Beta Hemolytic Streptococci in the Throats of Normal and Discased Human Beings

-11/0	Norn	ral and Dis	cased H	e Beta Iuman	Hemolytic Beings	Streptococc
Status of subjects	No. of subjects in series	of Culture positive f	or Dosi	ultures itive for inute otococci	Subjects positive for	minute for
Acute tonsillitis	1 ~ 1 1	No.   Pe cen   44   11   13   4   4   4   16   13   75   68   37   100   22   24   12   25   03   34   1	No.  24 37 4 10 4 3 41 4 2	Per	$\begin{bmatrix} 29 & 73 \\ 4 & 4 \\ 5 & 14 \\ 64 & 2 \end{bmatrix}$	Streptococci
A murviduals (11 per	- cocci M	ere found	in 41.	_		

individuals, minute streptococci were found in the throats of 44 of

A second group of 40 normal individuals was studied at weekly intervals during the first 3 months of 1934. As will be seen in Table II, 471 throat cultures were obtained and of these 37 (8 per cent) vere positive for minute streptococci while 61 (13 per cent) were posiive for ordinary beta hemolytic streptococci. In this group 9 (23 per ent) individuals showed the minute organisms at one time or the her and 29 (73 per cent) were positive one or more times for ordinary molytic streptococci. It is interesting to note that of the 9 indi-

TABLE II

Incidence of Beta Hemolytic Streptococci and of Minute Beta Hemolytic Streptococci in the Throats of 40 Normal Individuals as Determined by Weekly Throat

Cultures during a Period of 12 Weeks

- Cutures during & 1 cross of 12 W cons												
	Results of cultures											
Subject	ist. wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.	9th wk.	10th wk	11th wk	12th wk.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38	WE	M BM B B B	3rd wt.		5th wk.	B — — — — — — — — — — — — — — — — — — —		M B B B B B B B B B B B B B B B B B B B	9th wk.	B B M B B B B B B B B B B B B B B B B B	M B H H H H H H H H H H H H H H H H H H	B B M B B M M M M M M M M M M M M M M M
39 40	—	BM	B —	<u> </u>	-	-	_ n	— м —		B	B	_

B = beta hemolytic streptococci.

M = minute hemolytic streptococci.

<sup>- =</sup> negative for minute and beta hemolytic streptococci.

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TABLE III  Seta Hemolytic Streptococci in Throat erular Nephritis  Results of cultures (March, 1933, to July, 1934)  6 7 8 9 10 11 11  M M M M B B B B B B B B B B B B B B B	1 1 1 1
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N   W   B   W   W   B   W   W   B   W   W	Ka kan
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Occurrence of Bela Hemolytic Streptococci and Minute Bela Hemolytic Streptococci in Throat Cultures from Patients III with Glomerular Nephritis.  Subject Status   Acute M M M M M M M M M M M M M M M M M M M	
β <sub>th</sub>   γ <sub>α</sub>	
0 Si. Si. KI. KI. KI. KI. Mo.	We. Mu. Fo. Me. C. S. An. Pai. We. Ac. Pr. Bi. Ma. Ma.
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viduals in whom the minute organisms were demonstrated, 5 showed definite evidence of chronic infectious processes in the lymphoid tissue of their rhinopharynges.

In a group of ward patients suffering from essentially chronic disease, e.g. carcinoma, diabetes, hypertension, 102 single cultures were made from 102 individuals. Only 4 of these cultures (4 per cent) were positive for minute hemolytic streptococci and the same number showed ordinary hemolytic streptococci. Studies of the throat flora of individuals ill with acute respiratory tract affections in which the hemolytic streptococcus does not generally play an important rôle, for instance diphtheria, lobar pneumonia, influenza, colds, measles, hay fever, revealed a low incidence of the organisms, for only 9 (8 per cent) of 108 subjects in this group yielded minute hemolytic streptococci, while 15 (14 per cent) harbored the ordinary hemolytic streptococci.

Studies of the throat flora of individuals who were ill with diseases of proven streptococcus etiology, for example scarlet fever and septic sore throat, showed that these diseases were not associated with a high incidence of minute hemolytic streptococci, a fact demonstrated in Table I. 117 throat cultures were obtained from 110 individuals ill with scarlet fever and only 4 (about 4 per cent) cultures were positive for minute hemolytic streptococci. 75 (68 per cent) cultures showed ordinary beta hemolytic streptococci. These last figures on the incidence of bela hemolytic streptococci in scarlet fever may seem somewhat low, but as the cultures were obtained from the 1st to the 21st day of the disease, the high incidence of hemolytic streptococci found when cultures are taken during the first days of scarlet fever would not be expected. During the past year we were able to obtain 37 throat cultures from 22 individuals who were suffering from acute follicular tonsillitis. All of the cultures were positive for ordinary beta hemolytic streptococci, while 3 (8 per cent) cultures from 3 (15 per cent) individuals showed minute streptococci.

A group consisting of 59 individuals suffering from acute rheumatic fever or from rheumatic heart disease was studied for the presence of beta hemolytic streptococci in their rhinopharynges. cent) of these individuals minute hemolytic streptococci were found, while only 18 (31 per cent) patients were positive for ordinary hemoly-In 25 (43 per

tic streptococci. As no attempt was made in this series of patients to correlate the degree of activity of the rheumatic process with the time of the throat cultures, the relatively high incidence of the minute hemolytic streptococci is of interest.

Our final studies were made upon a group of 61 individuals who either had had glomerular nephritis and had recovered or who were suffering from either acute, latent or progressive glomerular nephritis at the time the investigations upon their throat flora were conducted. Of the 61 individuals 19 were considered as being well and in this group minute streptococci were found once in each of 2 individuals, while ordinary hemolytic streptococci were found in 9 individuals in this group.

Table III shows the results of 274 throat cultures obtained from 42 individuals who have been under observation because of the presence of an acute, latent or progressive glomerular nephritis during the past 14 months. From these 42 individuals we have secured 108 (36 per cent) cultures which were positive for minute streptococci and 103 (34 per cent) cultures which showed ordinary beta hemolytic streptococci. The minute streptococci were found in 33 (79 per cent) of the members of this group, and in 14 (33 per cent) of the patients they were the only hemolytic streptococci isolated during the period of investigation. Ordinary beta hemolytic streptococci were found alone in 4 (10 per cent) members of this group, while 19 (45 per cent) individuals showed both organisms in their throat cultures at various times during the period of study. In 5 (12 per cent) individuals neither representative of the hemolytic streptococcus group was found during the course of our investigation.

### DISCUSSION

Minute hemolytic streptococci were infrequently isolated from the throats of a large group of normal individuals, from whose throats only single cultures were taken. However in a smaller group of normal individuals in whom an investigation of the throat flora was made at weekly intervals over a period of 3 months, the organisms were found to occur more frequently although their incidence was considerably lower than that of the ordinary beta hemolytic streptococcus.

In individuals who were suffering from a variety of chronic diseases

the incidence of both ordinary and minute beta hemolytic streptococci was low and the same was essentially true of individuals ill with acute respiratory tract affections of non-streptococcal origin. organisms were infrequently isolated from the throats of patients who were suffering from scarlet fever or severe acute tonsillitis. The minute lent diseases we have on three occasions isolated the minute organisms in pure culture—once from an abscess in the deep tissues of the chest wall and twice from acutely inflamed paranasal sinuses, thus showing that they are capable in themselves of producing infections.

These minute hemolytic streptococci have been isolated most frequently from the throats of individuals who were suffering from glomerular nephritis or from chronic rheumatic infection. the well known association of ordinary beta hemolytic streptococci with the progression of these diseases we believe that our findings assume added importance because in 14 of the nephritis group and in 17 of the rheumatic group, minute beta hemolytic streptococci were the only members of the hemolytic streptococcus family isolated during the period of investigation. Possibly a careful examination of throat cultures from all subjects ill with these two diseases will eventually reveal all of them to be chronically infected with one or the other type of beta hemolytic streptococcus. We feel that the burden of proof, especially in regard to the association of hemolytic streptococci with the progression of glomerular nephritis, rests upon those who state that evidence of beta hemolytic streptococcal infestation is lacking in this group of patients.

### CONCLUSIONS

- 1. Minute beta hemolytic streptococci were found to occur from one-third to one-half as frequently in normal individuals as do ordinary
- 2. They were rarely isolated from the rhinopharynges of individuals suffering from chronic disease.
- 3. In acute respiratory tract infection other than that due to the ordinary beta hemolytic streptococcus the incidence of minute streptococci was slightly higher than in normal individuals.
- 4. In acute streptococcal infections, scarlet fever and acute tonsillitis, for example, the incidence of minute hemolytic streptococci did

not significantly vary from the incidence found in normal human beings.

- 5. Minute beta hemolytic streptococci were found in the throats of 33 out of 42 patients ill with glomerular nephritis and in 25 out of 59 patients who were suffering from the various manifestations of rheumatic fever.
- 6. In glomerular nephritis and rheumatic infection the minute beta hemolytic streptococci were isolated from the throats of more patients than were the ordinary beta hemolytic streptococci.

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#### CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION\*

#### T. A METHOD

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Analytical methods in immunology have rarely approached in accuracy the procedures which the chemist is willing to consider as quantitative analyses. This has been due in great measure to the lack of knowledge of the nature of the materials between which immune reactions take place, so that it has been necessary to consider only "units" of reactivity and to arrive at some conclusion regarding these by dilution methods. The failure to use calibrated pipettes and the uncertainty as to whether the final reading should be, for example, + or  $\pm$ , render methods of this type subject to very large errors which are increased in the case of bacterial agglutination by uncertain methods of standardizing the cell suspensions.

In recent years a mass of evidence has accumulated which can be satisfactorily interpreted only on the basis that immune reactions are chemical reactions. The chemistry of the components of these reactions, namely antigens or haptens on the one hand, and antibodies on the other, is now much more fully understood. While few of these substances have been isolated in a state of absolute purity, enough is known to permit the formulation of methods which fulfill the requirements of quantitative analytical chemistry and allow the expression of the result not merely in relative terms but in actual mass units such as grams per liter, or in milligrams per cubic centimeter. The validity of these methods rests on the assumption that antibody is actually protein. The evidence for this assumption has been reviewed elsewhere (1, 2).

<sup>\*</sup>The work reported in this communication was carried out under the Hark-ness Research Fund of the Presbyterian Hospital.

On this basis a method has been developed in this laboratory for the quantitative micro-determination of precipitins (3-5). It was first necessary to establish the conditions for precipitating the maximum amount of antibody (6), the result being that the method gives directly the maximum number of milligrams of specifically precipitable antibody per cubic centimeter of serum. The method can also be reversed and used for the quantitative micro-determination of an antigen or hapten (4).

The agglutination of bacteria may best be interpreted as a precipitin reaction at the surface of the cells (7, 8). It therefore appeared possible that a suitable modification of the precipitin method would provide an absolute method for the micro-determination of agglutinins.

The method and its application in several instances are described in the present communication, while the papers that follow will deal with the results obtained and their bearing on the mechanism of bacterial agglutination.

## EXPERIMENTAL

- 1. Preparation of Bacterial Suspensions.—The microorganisms studied were grown for 16 to 24 hours in broth containing 0.4 per cent of phosphate and 0.1 per cent of glucose for S organisms and 0.25 per cent of glucose for R organisms. The cells were centrifuged off, suspended in about 200 cc. of saline and killed by heating at 60°C. for 45 minutes or by adding formalin to a concentration of 0.5 per cent and allowing to stand at room temperature or at 37° for 72 hours. The cells were then washed repeatedly with 0.9 per cent saline or saline containing 0.025 per cent formalin if the suspension was formalin-killed until the supernatant no longer gave a biuret test and nitrogen analyses on 3 to 5 cc. gave results within the limit of error of the micro-Kjeldahl method (0.01 to 0.02 mg. N). At least seven washings were carried out. After each centrifugation the supernatants were drained as completely as possible. The washed cells were finally suspended in a volume of saline such that each cubic centimeter of suspension contained 0.15 to 0.25 mg. of nitrogen. Merthiolate was added in a final concentration of 0.01 per cent and the suspension was kept in the refrigerator.
- 2. Antisera.—The antisera used were antibacterial rabbit and horse sera. One of the latter was a Type I, II antipneumococcus horse serum which had been deprived of its Type II, protein, and C antibodies as fully as possible by absorp-

A preliminary note was published in Proc. Soc. Exp. Biol. and Med., 1934, 31, 595 (9).

tion respectively with Type II pneumococcus specific polysaccharide and with C polysaccharide and protein derived from Type I R pneumococci. The other immune horse serum used was a Type I antipneumococcus serum kindly furnished by Dr. William H. Park of the New York City Department of Health laboratories. It was freed as completely as possible from antiprotein and anti-C as above, except that C substance derived from Type III was used. The remaining Type I anticarbohydrate in each serum was then purified according to Felton (10). The resulting antibody solutions were designated as B 75 and B 76 respectively.

3. Analytical Procedure.—A number of determinations may be run at one time. From 0.5 to 2.0 cc. of serum, depending on the potency of the serum, are added in duplicate from calibrated pipettes to Wassermann tubes. 2 to 3 cc. of the uniformly mixed bacterial suspension are added to each sample from a calibrated pipette. Blanks containing the bacterial suspension and saline instead of serum are set up at the same time, as is also a salt control consisting of serum plus salt with no addition of bacteria. The contents of the tubes are then thoroughly mixed by repeatedly drawing the finger-tips rapidly and diagonally down the sides of the tubes. The tubes are placed in the water bath at 37° for 2 hours and then in the ice box overnight, or the experiment may be conducted entirely at 0° and left for 24 or, better, 48 hours, with occasional mixing. Visible agglutination usually occurs immediately if the serum is fairly strong, and if any agglutinin is present, the serum tubes should appear more turbid than the blanks. If the bacteria flocculate, the contents of the tubes are gently mixed as above every 15 or 20 minutes while in the water bath to aid in the establishment of equilibrium. After standing overnight the tubes are centrifuged in the refrigerating centrifuge<sup>2</sup> at 2,000 R.P.M. The blanks in some instances were run at 2,800 R.P.M. in a small Swedish angle centrifuge3 placed in the ice box and in other cases at 2,800 R.P.M. at room temperature, since the unagglutinated organisms were more difficult to centrifuge down tightly. The supernatants are completely decanted and the tubes allowed to drain provided the deposit shows no tendency to run down the sides. In many instances, particularly in the case of the blanks, repeated centrifugation is necessary to obtain a well packed deposit of bacteria. If purified antibody solutions are used aliquot portions of the supernatants may be analyzed for nitrogen by the micro-Kjeldahl method and the value obtained subtracted from the total nitrogen of the antibody solution as an additional check.

After draining the tubes are placed in ice water. 0.5 cc. of cold saline is added to each and the precipitates are well broken up and resuspended. The tubes are then rinsed down with 2.5 cc. of cold saline and the contents again mixed. The tubes are allowed to stand in ice water for ½ hour and centrifuged again in the cold. The supernatants are again decanted, the tubes drained and the pre-

<sup>&</sup>lt;sup>2</sup> Manufactured by the International Equipment Co., Boston.

<sup>&</sup>lt;sup>3</sup> Supplied by the Standard Scientific Supply Co., New York.

cipitates washed a second time in a similar manner, after which the supernatants are decanted and the tubes drained. The precipitates are then suspended in water and quantitatively transferred to micro-Kjeldahl flasks with the aid of The micro-Kjeldahl analysis for nitrogen is then carried out in the usual way except that it is convenient to run the digestion in 100 cc. pyrex Kjeldahl flasks. It is advisable to pour off the supernatants separately into marked tubes, for if additional centrifugation is necessary. After the minute deposit which is thus the main tube corresponding with it may be poured upon it, rinsing down the second washing from the main tube is added, and after this has been centrifuged, the two deposits are transferred to a micro-Kjeldahl flask.

Milligrams agglutinin N, for the volume of serum used, = N determined - N in bacterial suspension blank.<sup>4</sup>
Agglutinin  $N \times 6.25 = \text{agglutinin}$  in milligrams for the volume used.

The agglutinin content of a serum in milligrams per cubic centimeter is the maximum value obtained by dividing the agglutinin found by the number of cubic centimeters used. If this value is desired the amounts of serum and suspension should be adjusted so that antigen is in excess. The method is checked by setting up aliquot portions of the supernatants with a second portion of the bacterial suspension. If the value for nitrogen obtained is the same as that in number of milligrams per cubic centimeter equals the total agglutinin content. If a small additional amount of nitrogen is precipitated in the determination on first value.

The method in its present form is not applicable to antisera to Streptococcus hemolyticus, owing to the dense suspensions and repeated absorptions necessary to exhaust the sera. In the case of pneumococcus it has also been found very difficult to use heat-killed R (or "S" according to Dawson's terminology (11)) suspensions because of the formation of surface films of bacteria which are easily decanted and must be recentrifuged as noted above. Formalinized R suspensions killed suspensions may be used.

In Table I are presented data showing the application of the method under various conditions to Pneumococcus I S (Dawson "M") suspensions and homologous purified antibody obtained from horse sera according to Felton (10).

If the serum-salt control shows any sediment it should be washed as above, analyzed for N and its N content also subtracted from the total N.

cus I S (Dawson "M") Suspensions in Homologous Horse Antibody Solutions B 75 and B 76 TABLE I ć

	Analyses on supernatants	M lenoT  Macterial M  M  Macterial M  M  M  M  M  M  M  M  M  M  M  M  M	mg. mg. mg. mg. ms. ms. ms.	0.81 0.63 0.18 1.80	1.52 0.63 0.89 1 86	0.64 0.61 0.03}	0.71 0.20 0.51 1.37 0.87	0.20 0.33   1.37 1.04 0.33	1.90 1.35 0.42 0.93	0.60 0.96 1.92 1.35 0.40 0.95	1.53 0.60 0.93 1.86 1.35 0.43 0.92 1.84	1.06 0.43 0.63	0.47 0.43 0.04\$	1.08 0.43 0.65	0.46 0.43 0.03	1.05 0.42 0.63	1.30
Somon 11	<u> </u>	Temper- atures used	25	*	37 & 0	37 & 0 (	37 & 0 (		-		•	0	0	0	0	37 & 0 1	
Agglutination of Pneumococcus I S (Dawson "AL") Suspensions in Bonnorekons		Period in refrigerator	hrs.	Overnight	3	<del></del>	3	=	36	88	36	Overnight	48	48	48	Overnight	
W. 110.		Total volume	9	2.50	2.50	4.0	4.0	0,4	3.5	3.5	12.0	4.0	5.5	4.0	5.5	2.5	
S (Datus		Labo- ratory designa- tion of suspen- sion		υ	ບ	υ	<u></u>	) F	Ω	D	a	Þ	ন	ಟ	ĸ	£	;
occus I .		Volume of pneumococcus suspension used	;	2.00	2.00	2.00	8	9	3.00	3.00	3.00	2.00	2.00	2.00	2.00	2.00	
n of Pueumoc		Volume of	,	0.50	0.50	{2 cc. super- natant	Ç	05.0	0.50	0.50	0.50	0.50	3.5 cc. supernatant	0.50	3.5 cc. su-	0.50	7.0 cc. su-
Agglutinatio		Antibody solution		B 75 (1:5)	B 75					<del></del>		11,76	<u>- , </u>				

\*2 hours at 37° and overnight in the refrigerator. † 0.04, corrected for aliquot taken. ‡ In this case represents refrigerator temperatures. § 0.05, corrected for aliquot taken. | 0.06, corrected for aliquot taken.

Data already published in the preliminary note (9) are omitted. With one or two exceptions each nitrogen value represents the average of duplicate determinations. The precision of the method is such that duplicates usually agree within 0.05 cc. of N/70 HCl or 0.01 mg. of N. The agreement of agglutinin N as calculated from analyses both of precipitate and supernatant eliminates the possibility of errors in washing the precipitates such as incomplete removal of non-specific N or loss of bacterial sediment. Analyses of the entire second washings in experiments with horse Serum 610 in which 60 or 70 mg, of non-specific protein were originally present yielded values of 0.00 and 0.01 mg. N, showing that two washings are adequate, as in the precipitin reaction. A determination using a suspension of pneumococci and an anti-egg albumin serum gave recovery of only the bacterial nitrogen (cf. 9), again showing that non-specific protein is not held back. If a value for agglutinin N/cc. is given in the table, the antibody was completely removed, as shown by an analysis of the supernatant with a further addition of bacteria. This was, however, omitted in the experiments with 3.0 cc. of Suspension D, since the excess of pneumococci was large and the results agreed with previous total antibody N determinations on the same solution.

The effects of making the analyses at different temperatures and dilutions are shown in the experiments with Suspension D and antibody Solution B 75. The first two sets of analyses show that, in the region of excess antibody, reaction is not complete in 24 hours at 0°, while the remaining analyses indicate that, provided antigen be present in excess, maximum figures for antibody N may be obtained at 0° (possibly using a longer period of interaction than 24 hours) as well as under the ordinary conditions. As for dilution, the values obtained in any one series of experiments, while scarcely varying outside of the range of accuracy of the method, might be taken to indicate that 0.01 to 0.03 mg. of antibody N may be lost if the analysis is not carried out in as small a volume as possible. As a result of these experiments the standard conditions for the determination of agglutinin N adopted in this laboratory involve the use of low dilutions and interaction of the components at 0° for 48 hours. It will be noticed from Table I, however, that antibody Solution B 76 gave the same results at 37° and 0°, and at 0° for 24 and 48 hours, assuming all antibody to have been removed after the second absorption.5

In Table II are given data obtained in the reaction between Pneumococcus I S suspensions and horse and rabbit antisera of homologous type. The horse serum was the one from which antibody Solution B 76 (Tables I and III) was prepared. Rabbit Serum 252 in the raw state (A) contained complement, although much less than the guinea pig sera ordinarily used. After inactivation in a stoppered tube for 45 minutes at 56° (B) the agglutinin content for Pneumococcus I S organisms was unchanged, indicating that absorption of a small amount of complement, as in the A series, does not increase the amount of nitrogen precipitated within the limit of accuracy of the method used. An attempt will be made to repeat this experiment with sera of higher complement content.

<sup>&</sup>lt;sup>5</sup> Later experiments have justified this assumption.

It will be noted that repeated absorptions of the rabbit sera are necessary in order to remove all the agglutinin. These sera were not absorbed with pneumococcus C substance or protein or R organisms, so that it is possible that the

TABLE II

Agglutination of Pneumococcus I S (Dawson "M") Suspensions in Homologous

Horse (H) and Rabbit (R) Antisera

		Volume		Analyse	Aggiu-		
Serum	Volume of serum used	of suspen- sions	Suspen- sions	Total N	Bacte- rial N	Agglu- tinin N precipi- tated	tinin N per cc.
	æ.	α.		rig.	mg.	r:z.	mg.
H 610	(0.50	2.00	D	1.17	0.41	0.761	
	2.0 cc.					}	1.56
	supernatant	2.00	D	0.43	0.41	0.02	
R 252	(0.50*	2.00	E	0.77	0.42	0.35	1.06
A	2.5 cc. supernatant	1.50	Ē	0.39	0.33	0.06f	1.00
	3.5 cc. 2nd	1				]	
	supernatant	1.50	E	0.38	0.33	0.05†	
	Entire 3rd						
	supernatant	1.50	E	0.36	0.33	0.03‡	
R 252	(0.50*	2.00	E	0.78	0.42	0.36	
В	2.5 cc. supernatant	1.50	E	0.39	0.33	0.06†	}
	3.5 cc. 2nd			1	1		į
	supernatant	1.50	E	0.38	0.33	0.05†	•
R 271	(0.50*	2.00	E	0.86	0.42	0.44	0.98
	2.5 cc. supernatant	1.50	E	0.35	0.33	0.02	0.50
	3.5 cc. 2nd						1
	supernatant	1.50	E	0.35	0.33	0.025	į
	Entire 3rd	1	1		1	_	}
	Supernatant	1.50	E	0.33	0.33	0.00	ļ

<sup>\* + 0.50</sup> cc. saline.

small amounts of agglutinin N remaining after the initial agglutination are due to anti-C and antiprotein in the sera.

<sup>† 0.07,</sup> corrected for aliquot taken.

<sup>‡ 0.04,</sup> corrected for aliquot taken.

<sup>§ 0.03,</sup> corrected for aliquot taken.

In Table III are given data for the agglutination of Pneumococcus I and II R

Agglutination of Pneumococcus I and II R (Dawson "S") Suspensions in Type I Antipneumococcus Horse (H) and Rabbit (R) Antiscra

	Pheumococcus H.	Lawson Com
	1 1107	se (H) and Rabbit (R) Antiscra
		Type
Antibody or serum	1	i i i i i i i i i i i i i i i i i i i
or scrum	Volum	Vol. Analyses on angles
	Volume used	ume of Sugar bacteria
		suspen- duspen-
		Arch.
H 610		
11 010	(O ==	N precipie
1	$\{0.50\}$	cc.   tated
	2.0 cc. supernatant	2.00   T.D.   mg.   mg.   mg.
H 610	-Pernatant	
1	[0.50]	-
1		00
R 76.4	Supernatant /	00   TD*   _     "
B 76 (prepared	0.50	.00   Tp*   0.3/  0.44  0 12)
	0.30	1 1 2 2 0.4410 0.7 10 202
7 (	$2.0 \text{ cc. supernatant}$ $\begin{pmatrix} 2.0 \\ 2.0 \end{pmatrix}$	VIII That I I I I
B 75	Politatant   2.	00   50   0.55   0.46   0.5
[0	.50 l	1 1 10 60 0 - 0 0.09 1
12	.0 cc. supernatant 2.0	$0.09 \mid 0.69 \mid 0.00 \mid 0.18 \mid 0.18 \mid 0.00 \mid 0.18 \mid 0.18 \mid 0.00 \mid 0.18 \mid 0.00 \mid $
R 176	2.0	0 II R* 0 44 0 0 1
(Type II P 1.		0 II R* 0.44 0.34 0.10 0.30 0.30 0.30 0.30
(Type II R anti- $\begin{cases} 1 \\ 2 \end{cases}$		1 1 0 0 0.29 0 0.7 10 22
DCIUM 1 1-1	5 cc. supernatant 2.00	
( )	240	
1 1 Si	IDernat.	$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$
5.5	cc. $3rd$ 2.00	
/	pernatant	
R 176	2.00	$\left \begin{array}{c c} 1 & R & 0.32 & 0.28 & 0.04** & 0.26 \end{array}\right $
1.0	00	$\begin{array}{c c} IR \parallel & 0.30 & 0.28 & 0.02 \dagger \dagger \end{array}$
1 25	12001	[ 0.28] 0.02†† [
1 34.0	c. supernatant 2.00 2.00	11 R     0 Ar
, ,	. 240	TT D11 1 - 201 U. 1010 20 1 1
* TY	<sup>2</sup> Ωatan+	$\begin{bmatrix} 11 & R \parallel & 0.27 & 0.19 & 0.26 \\ 0.19 & 0.0811 & 0.0811 & 0.0811 \end{bmatrix}$
4.1CH F 19911 9		R   = 0.20  0.10  = 0.0341  0.37
10/ and 00		(1  R)
† 37° and 0° overnight	A 90-	1 - 1   0.01
§ New dilusi	A second set of de	
v = 1017 (1114.)	or de	Armaia.

<sup>†37°</sup> and 0° overnight. A second set of determinations gave 0.16 mg. § New dilution of stock suspension.

Killed with acid buffer at pH 4.

<sup>¶ 0.07,</sup> corrected for aliquot taken.

<sup>\*\* 0.05,</sup> corrected for aliquot taken.

<sup>†† 0.03,</sup> corrected for aliquot taken.

<sup>‡‡ 0.10,</sup> corrected for aliquot taken.

(Dawson "S") suspensions in homologous rabbit antisera and horse serum and antibody solutions. Although the antibody solutions were prepared from horse sera absorbed with C substance and pneumococcus protein until no further precipitates could be obtained they still contained considerable agglutinin for the R organisms. Probably the pneumococcus protein-antiprotein complexes are highly dissociated. They would therefore be incompletely precipitated, but could be removed more completely by successive portions of solid antigens such as Pneumococcus R organisms.

Noteworthy also is the greater amount of agglutinin nitrogen removed from Serum R 176 by the homologous II R organisms than by the I R cells. Since the serum gave no precipitate with Type II pneumococcus specific polysaccharide, it is probable that the difference, 0.11 mg. of agglutinin N per cc., was not due to any Type II specific polysaccharide in the cells and antipolysaccharide in the serum. If this be correct, further work must be done to determine whether the discrepancy is due to differences in the proteins of the organisms which have escaped detection by the qualitative methods hitherto used, or whether some other cause is operative.

#### DISCUSSION

The validity of methods such as the present one has been challenged by Topley (12) in the case of the precipitin reaction since they are not based on the optimal proportions principle (13). The writers are unable to accept the optimal proportions method as a standard for the following reasons: (1) in a precipitation or agglutination reaction the factors influencing the velocity are imperfectly understood; (2) the position of the flocculation optimum is not independent of the dilution and varies whether the antigen or antibody be diluted; (3) in neither case is the optimum at the point of antibody exhaustion (13, 14), so that the method gives no information as to the actual agglutinin or precipitin content of a serum. With these limitations the optimal proportions method serves as a convenient one for the comparison of sera when only approximate, relative values are desired, but it becomes extremely cumbersome when attempts are made to endow it with quantitative significance (cf. 15, 16).

The present method is simple and convenient in the case of Pneumococcus S (Dawson "M") organisms, but is more exacting and difficult in the case of R (Dawson "S") organisms, since these do not centrifuge as well. It affords an exact analytical determination of the total amount of agglutinin present in absolute, not relative terms.

and should therefore be useful in instances in which accuracy is demanded and scientific, not routine, data are sought. Thus it has already yielded evidence of the quantitative correspondence of agglutinin and precipitin6 and has afforded new data on the mechanism of bacterial agglutination. The method should be useful in determining the agglutinin content of standard sera, with which sera could then be compared by the commonly used relative methods.

The range of applicability of the method among the non-encapsulated bacteria is yet to be determined. Preliminary tests with an hemolytic streptococcus system were not encouraging, as complete absorption of the antisera required too large amounts of the heatkilled suspension used. Experiments are being continued with other types of streptococcus suspensions.

With the aid of the present method it is possible to estimate quantitatively the agglutinin content of a serum for different variants of the same organism. Thus Antiserum H 610 contains 1.56 mg. of agglutinin nitrogen per cc. for Pneumococcus I S organisms and only 0.28 mg. per cc. for Pneumococcus I R cells. This does not mean that the "titer" as measured qualitatively for S cells would be higher than that for R cells; on the contrary, it would probably be lower, since Serum R 176, containing 0.26 mg. of anti-I R per cc., agglutinates this organism at a dilution of 1:800, and anti-S sera with a "titer" of over 1:80 or 1:120 are rare. The quantitative data are rather an expression of the fact that Pneumococcus I specific polysaccharide, just as the Type III substance (3), can combine with 40 to 180 times its weight of antibody,7 while the ratios between pneumococcus protein and its homologous antibody are certainly much smaller. This not only explains why the most potent type-specific antipneumococcus sera are of comparatively low agglutinin "titer," although of high agglutinin content, but also makes readily understandable the large amounts of serum or antibody solution often required in therapeutic practice in pneumonia.

It is thus clear that agglutinin "titers" are often valueless even for comparative purposes unless the comparison be restricted to a single variant of a microorganism. The absolute method of agglutinin

determination, described in the present communication, is, however, free from misleading implications of this nature.

#### SUMMARY

- 1. A method, conforming to the criteria of quantitative analytical chemistry, is described for the estimation of the agglutinin content of antisera. Examples are given of the application of the method to various antipneumococcus sera.
- 2. This new, absolute method is discussed with regard to its relation to the commonly used relative methods.

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# THE PROTECTION AFFORDED BY VACCINATION AGAINST SECONDARY INVADERS DURING COLDS IN INFANCY

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(Received for publication, July 27, 1934)

Studies of the mechanism of epidemic respiratory diseases in a concentrated infant population1 previously reported (1) have indicated that the etiology of these disorders may be a complex one. It has been noted that respiratory infection occurring in the autumn, when the carrier rate of pathogenic bacteria is low, usually takes the form of a minor disturbance of the type of the common cold. Later on, in the winter months, when a widespread dissemination of pathogenic bacteria takes place in certain groups of infants, an outbreak of infections in these groups takes on a more serious character. The majority of these infections present two features in common, fever and constitutional reaction; thus they are no longer designated as colds, but rather as grippe, pharyngitis, otitis media, bronchitis, or pneumonia, depending on the region principally involved. During the past few years it has been established that there exists in adults suffering from the common cold (2-5) a typical filtrable virus. There is every reason to suppose that this virus is equally pathogenic for infants. It has therefore been possible to construct an hypothesis as to the mechanism of development of epidemic respiratory diseases in infants: the filtrable virus alone produces the mild autumnal disturbance, the common cold: later on, when the carrier rate of pathogenic organisms becomes very high, it acts as an initiating agent which permits these bacteria to produce more severe infections.

Because of the existence of this filtrable virus, it is illogical to suppose that vaccination of adults with the bacteria of the upper respira-

<sup>&</sup>lt;sup>1</sup> These studies were made at The Home for Hebrew Infants in New York City through the courtesy of the late Dr. Alfred F. Hess and other members of the staff.

tory tract would prevent the common cold, and this has been amply established by most of the well controlled experiments along this line (6-9). On the other hand, if our hypothesis in regard to the etiology of epidemic respiratory diseases in a dense infant population be correct, vaccination with the secondary invaders should diminish the severity of the febrile and complicated forms of these diseases. A study, therefore, was conducted with this in view.

The only record that has been found of the use of such vaccines in early infancy is included in a report by Stoltenberg (10). New admissions to a children's hospital in Oslo were treated with a bacterial vaccine, and of a group of 500 so treated during a 5 year period, approximately 50 were under 1 year of age. A similar number of infants in the preceding 5 year period were considered as con-The frequency of febrile infections was given as roughly five times greater in the non-vaccinated group, and the number of complications also larger.

Previous experience (1) had led us to the belief that pneumococcus, H. influenzae, and hemolytic streptococcus are the most important pathogenic bacteria of the upper respiratory tract in infancy. therefore decided to employ a vaccine composed only of these organisms, in the hope that its simplicity would render it a more effective antigen than the average "catarrhal" vaccine.

Pneumococci were cultivated for 24 hours in beef infusion broth. The washed organisms were taken up in salt solution with 0.2 per cent phenol and heated to 60°C. for 30 minutes. Because of the fact that a considerable variety of types is recovered from infants—and it seemed difficult at the time to include all of these—it was thought that the most one could hope to produce was a non-typespecific active immunity. Consequently, the Neufeld strain of Type I pneumococcus was employed. It has recently been shown, however, by Goodner and Stillman (11) that this non-type-specific immunity is low in rabbits when their resistance to dermal pneumococcus infection is quantitatively estimated.

The hemolytic streptococcus vaccine was similarly prepared, except that it was heat-killed at 60°C. for 1 hour. Four strains, supplied by Dr. M. H. Dawson, were employed, derived from scarlet fever, rheumatic fever, erysipelas, and rheumatoid arthritis respectively. Dr. Margaret Pittman of The Rockefeller Institute kindly provided us with two smooth strains of H. influenzae, Types A and B. These organisms were cultivated 24 hours in clear chocolate broth, washed, suspended in carbolized saline, and heated to 60°C. for 45 minutes.

A group of twenty-three infants at the Home was designated as the one for study, and a comparable group of twenty-three in identical surroundings as the

control. The average age of each at the beginning of the observation period was 6.4 months. Starting in the latter part of October, 1932, a course of nine subcutaneous injections of the vaccine was administered to the first group. The initial dose of organisms was 45 million, the final dose 750 million. The proportion of pneumococcus to *H. influenzae* to streptococcus in each mixture was 5:3:3 approximately. Early in February a second course of seven weekly injections was administered to the treated group. The initial dose in this course was 190 million, the final dose 1000 million.

Some local redness and induration were produced by the larger doses of vaccine. No febrile or constitutional reaction was ever observed. It is to be noted that the administration of vaccine was always omitted if the infant had a febrile respiratory infection at the time; in this way, three members of the vaccinated group missed nearly all of the second course. They were not excluded, however, on this account from the tabulation of the clinical history of the group.

#### RESULTS

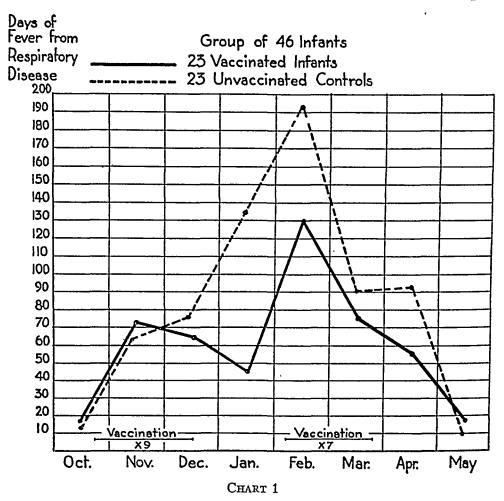
The two groups showed no significant difference in the incidence of minor respiratory infection of the type of the common cold.

The experience of each group in regard to the febrile forms of respiratory disease is graphically indicated in Chart 1. In this chart is given the total number of days of temperature over 100° due to respiratory disease for each month in the two groups. It will be observed that there was very little febrile infection during October. In November, half way through the first course of vaccine, the two groups fared almost identically. In December, also, the difference was slight. By January, however, there was a striking difference between the vaccinated and the control, the latter showing three times as many days of fever as the former. The peak of respiratory disease was reached in February, and in this month-half way through the second course—it will be seen that the difference between the groups is less marked, for the vaccinated infants showed more than half as many febrile days as the controls. There is a falling off for both groups in March, but it is to be observed that while this decline continues in the vaccinated group during April, it does not do so in the control. In May, both fell to their original low level.

The actual number of individual respiratory infections of all types per child—5.4 in the vaccinated, and 5.8 in the control—was almost the same in the two groups. The difference in severity, however, is further illustrated by the incidence of pneumonia; there were five cases

among the controls as opposed to two in the vaccinated, and of the latter one case occurred when the first course of vaccination was only half completed.

In spite of the fact that the N. Y. 5 strain of scarlatinal strepto-



coccus was included in the vaccine, two of the vaccinated children developed scarlet fever, as well as one control. There was evidence, however, that the causative streptococcus of this outbreak was an atypical one.

#### DISCUSSION

The data obtained in this experiment give evidence that vaccination of infants against certain bacteria of the upper respiratory tract modifies in degree the subsequent respiratory disease which these There is no alteration in the number of acute infants will undergo. infections, and no change in the simple catarrhal infections of the upper respiratory tract. There is, however, a significant alteration in the severity of respiratory infection, as measured by the number of days of fever that occur. The age group selected for study was the one in which our experience indicated that the greatest incidence and severity of respiratory infection might be expected during the winter months. Even in this age group which, in institutions, invariably shows so high a susceptibility to epidemic respiratory disease, vaccination with common pathogens is effective in reducing the severity of these epidemics. The interest here lies chiefly in such light as is thrown on the mechanism of epidemic respiratory disease. Earlier in this report an hypothesis was put forth that the character of certain winter outbreaks of grippe, etc., is determined by the presence of a widespread carrier state of ordinary pathogenic bacteria; the study herein described would seem to lend additional support to this hypothesis, inasmuch as vaccination with these pathogens has been found to modify significantly the severity of these outbreaks.

Certain other features of these results are worthy of brief comment. A study of the curve given in Chart 1 would seem to indicate that immunity in these infants did not manifest itself until a rather large amount of vaccine had been administered. It would also appear that the immunity finally engendered did not last much more than 2 months.

With the increasing data now available as to the frequency of the various newly identified types of pneumococcus carried in infancy, it seems probable that more striking results would have been obtained by the use of a vaccine containing these types instead of Type I.

#### SUMMARY AND CONCLUSIONS

An intensive course of vaccination with the pathogenic bacteria of the upper respiratory tract modified favorably the winter outbreak of severe respiratory disease in an infant population. The incidence of the common cold was not affected.

The significance of these findings is discussed.

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#### HEMORRHAGES IN SKIN LESIONS OF GUINEA PIGS FOLLOWING INTRAVASCULAR INJECTION OF TOXINS (SHWARTZMAN PHENOMENON)

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(Received for publication, August 1, 1934)

In 1924 Sanarelli (1) reported that rabbits infected with cholera vibrios react to B. coli or B. proleus in a way entirely different from that of normal rabbits. In a study on the virulence of cholera vibrios Sanarelli observed that in every large group of rabbits a few would die from the injection of minute doses, much smaller than the dose lethal for most rabbits. The symptoms resembled anaphylactic shock. In order to explain the mechanism of this phenomenon, Sanarelli studied the fate of cholera vibrios injected into the circulation. He found that the cholera vibrios disappeared from the blood stream within 2 days after their injection. But he also observed that in the rabbits which succumbed following the injection of sublethal doses, B. coli or proteus bacilli would invade the blood stream. Sanarelli now injected rabbits intravenously with minute doses of cholera vibrios and on the following day gave an intravenous injection of either cholera vibrios or of colon or proteus bacilli. Almost all of the rabbits died within from a few hours to 2 days after the second injection. The symptoms and pathological findings were the same for all three kinds of bacteria. Evidently the first injection of cholera vibrios prepared the rabbits, made them susceptible to a second, the injury-producing, injection of bacteria that by themselves were harmless.

In 1927 Shwartzman (2) reported his observations on the local skin reactivity to filtrates of *B. typhosus* cultures. He injected these filtrates into the skin of rabbits and introduced the same material 24 hours later into an ear vein. In the skin at the site of the injection a severe hemorrhagic necrosis appeared from 4 to 5 hours after the second injection. The intensity of the hemorrhagic necrosis did

<sup>&</sup>lt;sup>1</sup> The "preparatory" factor is the first, the intracutaneous, injection of toxin. The "injury-producing" factor is the second, the intravascular, injection of toxin. The terms "preparatory" and "injury-producing" factors or agents suggest that the preparatory factor does not cause injury. The experiments of Hanger and those reported in the present paper, however, show that the production of injury is essential in the action of the preparatory factor. These terms are employed in this publication because they are generally used in the literature.

not correspond to the severity of inflammation caused by the bacterial filtrate in the skin. Repeated injections into the same area of skin did not result in hemorrhagic necrosis. Turpentine or streptococcus filtrate did not prepare

One month after the publication of Shwartzman's report Hanger (4) published his studies. He observed that when a positive skin test with a filtrate from Bacterium lepisepticum is followed by the intravenous injection of the same material, an intensification of the local reaction with bluish discoloration and petechial hemorrhages occurs. Histological examination of the skin lesion showed intense swelling, disruption of the capillary endothelium, and minute thrombi in the blood The intensity of the hemorrhagic reaction is roughly proportional to the intensity of the inflammation caused by the intracutaneous injection of the filtrate. The hemorrhagic reaction is non-specific since inflammation of the skin induced by a filtrate from streptococci became hemorrhagic after an intravenous injection of a filtrate from B. lepisepticum.

The purpose of the present study is to inquire into the nature of the hemorrhagic reaction, particularly to ascertain whether this reaction is limited to the action of bacterial toxins or can also be produced by inflammatory irritants not related to bacterial toxins. In the present paper experiments on guinea pigs are reported.

# The Hemorrhagic Reaction in the Guinea Pig

The Shwartzman reaction in its original form has been studied mainly in rabbits. Gratia and Linz (5) reported that rats and mice are refractory and occasional guinea pigs are susceptible. Gross (6) never succeeded in producing the Shwartzman phenomenon in the guinea pig. In view of this contradiction, we injected twelve guinea pigs with filtrate from B. typhosus, found highly potent in rabbits. 0.2 cc. of the filtrates was injected into the skin and 1 cc. into the heart of the guinea pigs. None of them reacted with hemorrhage.

The Effect of Intravascular Injection of Bacterial Toxins upon the Specific Inflammation (Arthus Phenomenon) in Guinca Pigs

It was observed recently (7) that the intravascular injection of a filtrate from the culture of typhoid bacilli produces hemorrhagic necrosis in tuberculous guinea pigs at the site of tuberculin reactions. In normal guinea pigs the areas of skin injected with tuberculin were not affected by a subsequent injection of typhoid filtrate. Hence it is

probable that the inflammation produced by tuberculin in the hypersensitive tissue rather than products of the tubercle bacillus *per se* prepared the skin for the action of the typhoid toxin introduced into the blood stream.

In connection with this observation, the question arises whether injury produced by substances other than tuberculin would prepare the skin in guinea pigs. We have studied the action of various antigenic and non-antigenic substances, first of all that of horse serum in the sensitized guinea pig. It is interesting to compare from this point of view the tuberculin reaction with the Arthus phenomenon, for these two specific inflammations are different from each other in many respects.

The tuberculin reaction in guinea pigs is a "delayed" reaction (Zinsser (8)). It appears later than 12 hours and reaches its maximum usually only in 48 hours after the injection. In addition to redness and edema, sometimes purple discoloration is seen and the reaction often becomes necrotic. It cannot be transferred passively. In contrast to the tuberculin reaction, the reaction to horse serum (or to other proteins) is "immediate" (Zinsser). It appears within 2 hours after the injection and reaches its maximum often within 24 hours. It is characterized by redness and edema, purple discoloration and necrosis being conspicuously absent. It can be transferred with immune serum rich in precipitins. Zinsser pointed out that the two types of skin reaction, immediate and delayed, can be distinguished in the guinea pig but not in the rabbit.

We sensitized five guinea pigs to horse serum by injecting 0.1 cc. of horse serum into the subcutaneous tissue three times, 3 days apart. The guinea pigs were tested several times with various dilutions of horse serum. 1 day after the injections of 0.1 and 0.01 cc. of horse serum, redness and swelling was noted at the sites of the skin tests. Hemorrhage was not produced in any of the guinea pigs by the intracardial injection of typhoid filtrate. In guinea pigs sensitized to horse serum, therefore, the specific inflammation in the skin did not act as a preparatory factor.

The Effect of Intravascular Injection of Typhoid Toxins upon the Inflammation Caused by Diphtheria Toxin:

The inflammation that is produced by diphtheria toxin injected into the skin and the tuberculin reaction are similar in many aspects.

Both reactions develop slowly, reach their maximum after 2 days, and may be hemorrhagic and necrotic. This similarity suggested that diphtheria toxin might act as a skin preparatory agent.

TABLE I

Diphtheria Toxin as Skin Preparatory Agent in the Guinea Pig

Guinea pig No.	Preparatory agent, 0.1 cc. diphtheria toxin	Injury-producing agent, typhoid toxin	Hemorrhagic reaction
1	1:250 1:500	cc. 1	Present
2	1:250 1:500	0.5	· ·
3	1:400 1:800	1	Absent
4	1:400 1:800	0.5	· ·
5	1:400 1:800	0	Hemorrhagic before injection of typhoid toxin
6	1:500 1:750	1.5	Hemorrhage increased after the injection of typhoid toxin
7	1:500 1:750	1.5	Present
8	1:1,000 1:5,000 1:25,000	1	Absent
9	1:1,000 1:5,000 1:25,000	1	"

In the experiments with diphtheria toxin, nine guinea pigs were employed. The strength of this diphtheria toxin<sup>2</sup> was 0.0016 cc. 1 M.L.D., 0.15 cc. L+ dose. The dilutions of diphtheria toxin ranged from 1:250 to 1:25,000. Eight of the

<sup>&</sup>lt;sup>2</sup> Obtained through the courtesy of Dr. J. Reichel of the H. K. Mulford Co.

guinea pigs received intracardial injections of a typhoid filtrate 1 day after the intracutaneous injections of diphtheria toxin; one of the guinea pigs was injected with diphtheria toxin alone.

Table I shows that in two guinea pigs, Nos. 5 and 6, the reactions to the toxin were hemorrhagic, even without the subsequent injection of a bacterial filtrate. One of these two animals, No. 6, reacted with an increase of hemorrhage after the intracardial injection of typhoid filtrate. The other seven guinea pigs showed no purple discoloration 1 day after the injection of diphtheria toxin. When the guinea pigs received intracardial injections of from 0.5 to 1.5 cc. of typhoid filtrate, four of six of those prepared with toxin dilutions varying from 1:250 to 1:750, and none of those injected with toxin dilutions from 1:1,000 to 1:25,000, reacted with hemorrhage. Diphtheria toxin in the guinea pig acts as a skin preparatory factor.

Turpentine, Broth, and Silver Nitrate as Preparatory Factors

Three sterile inflammatory irritants, namely turpentine, concentrated broth, and silver nitrate, non-antigenic in nature, were examined as to their capacity to act as skin preparatory agents.

A 5 per cent solution of turpentine in paraffin oil was injected into the skin of nine guinea pigs. On the day following the injections redness, edema, and necrosis were found in all of them. The site of inflammation did not become hemorrhagic after the intracardial injection of typhoid filtrate.

Negative results were also obtained in experiments with glycerine broth control for tuberculin. This material is prepared by evaporating the glycerine broth used for growing tubercle bacilli to one-tenth of its original volume. Four guinea pigs were injected intracutaneously with various dilutions of glycerine broth control. No hemorrhage was observed after the injection of bacterial filtrate. The observations with turpentine and broth are in harmony with those of Hanger and Shwartzman, in rabbits.

Four control guinea pigs received only intracutaneous injections of silver nitrate solutions. At the site of the injections, gray-green necrosis, surrounded by redness and edema, was observed on the following 2 days. In one of the four animals the area of necrosis was bordered by a sharply defined line of hemorrhage 0.5 mm. wide. The hemorrhagic line appeared 1 day after the injection of silver

TABLE II
Silver Nitrate as Skin Preparatory Agent in Guinea Pigs

		P	are as S	kin Pre	barato;	ry Agen	t in Gui	nca Pigs
Guinea	Guinea pig No.		Preparatory agent, 0.1 cc. silver nitrate		Injury-producing agent, typhoid toxin		orrhagic action	
				cc.				Systemic reaction
1		1:100 1:200	) [					
	- 1	1:1,0		2.5		Pres	sent	Died in 24 hrs.
•		1:100			- 1		- 1	- 21 ms.
2	- 1	1:200	- 1	2.5	- 1		- 1	
		1:1,00	0	2.3	- 1	Abser	nt	
3		1:100*					- 1	
3	- 1	1:200*	- 1	2	- 1	Preser	nt	
1	- 1	1:1,000	)*	2	- 1	Absen	t	
,	- 1	1.100	- 1		- 1	"	- 1	
4	- 1	1:100 1:200	- 1		- 1		- 1	
	- 1	1:1,000		1	- 1	"	- 1	
	- 1	-11,000	- 1				- 1	
5	- 1	1:100	- 1		- [		- 1	
J	- 1	1:200	- 1	1	-		- 1	
	- 1	1:1,000	- 1	•	1	Present	- 1	
6	-   -	1:20	- [		1		- 1	
U		1:200	1	1	1		- 1	
			1	•	4	Absent	- 1	
7		:200	-				- 1	
,		:400	1		ח	resent	- 1	
	1	:1,000	1	ı	_	resent	"	" 24 "
8	1.	50		- 1			1	
U	,	200	l o.	75			1	
	1		1	.	Ab	sent	1	
9		500		- 1			1	
	1:1	,000	0.,	5	Prc	sent	1	
10	1:5	n 1		- 1		,	1	
"	1:20		0.5	- 1		- 1		
The pecret					"	- 1	" "	24 "
The necrot	tion of	was surr	ounded	by a pu	rnle ?			

<sup>\*</sup> The necrotic area was surrounded by a purple band about 0.5 mm. wide before the injection of typhoid toxin.

nitrate and did not increase in size during the following day. Ten guinea pigs were injected intracutaneously with 0.1 cc. of various dilutions of silver nitrate solution and intracardially with typhoid toxin. The guinea pigs reacted at the site of injection of silver nitrate with gray-green necrosis surrounded by redness and edema. In one guinea pig the necrotic area was surrounded by a purple band 2 mm. wide and a subsequent injection of bacterial filtrate increased the hemorrhage. The area of the hemorrhagic reaction in guinea pigs prepared with silver nitrate was less extensive than in those prepared with tuberculin or diphtheria toxin. In three animals it appeared in the form of a band from 2 to 3 mm. wide, surrounding the area of necrosis, but in two other guinea pigs the hemorrhages extended over areas of 30 x 30 mm. The injection of typhoid filtrate had no effect on the skin in four, and very slight effect in one guinea pigs. Three of these animals died following the injection of typhoid filtrate and hemorrhages in the spleen and effusion in the peritoneal cavity were found (Table II).

#### DISCUSSION

The experiments described in the present papers show that the conditions necessary for the hemorrhagic reaction, the Shwartzman phenomenon, are different in the guinea pig and the rabbit. According to our experiments which confirm those of Gross (6), but do not support entirely the conclusions of Gratia and Linz, bacterial filtrates potent in the rabbit are inactive as skin preparatory agents in the guinea pig.

It was found that the intravascular injection of typhoid toxin produced hemorrhage at the site of inflammation caused by diphtheria toxin or silver nitrate. Both of these irritants, namely diphtheria toxin and silver nitrate, elicit, like tuberculin in tuberculous guinea pigs, hemorrhagic necrosis in the skin of some of the guinea pigs even without the subsequent intravascular injection of a bacterial toxin. Arthus phenomenon with horse serum, turpentine, or broth did not act as a skin preparatory agent. It is noteworthy that those irritants that prepared the skin for the hemorrhagic reaction were capable of producing hemorrhages and necrosis in the skin of some of the guinea pigs, whereas horse serum (in guinea pigs sensitized to it), turpentine, and broth lacked both the capacity of causing hemorrhage and that of acting as skin preparatory agents. The capacity to elicit hemorrhage in the skin without subsequent intravascular injection of a toxic substance may be essential for the action of skin preparatory agents. The experiments with diphtheria toxin and silver nitrate show that the skin of guinea pigs can be successfully prepared for the hemorrhagic reaction by a true exotoxin and by a simple inorganic compound as

The experiments described and histological observations (to be published later) seem to support the following explanation of the Shwartzman phenomenon. The substance injected into the tissue at the first injection causes an injury at the site of injection, particularly to the blood vessels of that region. The injury is a transient one. The material introduced into the vascular system by the second injection augments the injury, resulting in rupture of blood vessels, hemorrhage, and thrombosis.

- SUMMARY AND CONCLUSIONS 1. Filtrates from B. coli, B. typhosus, or meningococci injected into the skin of guinea pigs do not produce visible inflammation. When these injections are followed by intravascular injections of the same material, hemorrhages do not occur in the skin.
- 2. Guinea pigs sensitized to horse serum react with redness and edema to 0.1 or 0.01 cc. of horse serum injected into the skin, and subsequent intravascular injection of typhoid filtrate does not produce hemorrhage at the site of the reaction to horse serum.
- 3. When guinea pigs are injected into the skin with diphtheria toxin and these injections are followed by intravascular injection of filtrates from B. typhosus, hemorrhage occurs in the skin at the site of the reaction to diphtheria toxin.
- 4. When silver nitrate is injected into the skin of guinea pigs, redness, edema, and necrosis follow, and in a few guinea pigs small areas of hemorrhage can also be noticed. About half of the guinea pigs that have received an intravascular injection of typhoid filtrate react with hemorrhage at the site of the injection of silver nitrate.

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# HEMORRHAGES IN TUBERCULOUS GUINEA PIGS AT THE SITE OF INJECTION OF IRRITANTS FOLLOWING INTRAVASCULAR INJECTIONS OF INJURIOUS SUBSTANCES (SHWARTZMAN PHENOMENON)

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In the preceding paper it was reported (1) that the intravascular injection of toxins of B. typhosus, B. coli, or meningococcus produced hemorrhages or hemorrhagic necrosis in the skin of guinea pigs at the site of reaction to diphtheria toxin or silver nitrate. Furthermore, it was described that similar injections of toxins failed to cause hemorrhages in the skin of guinea pigs at the site of injections of other irritants such as horse serum in guinea pigs sensitized to it, or turpentine or broth.

Recently, I have reported very briefly the observation that in a tuberculous guinea pig the skin becomes hemorrhagic at the site of positive tuberculin tests when products of typhoid bacilli are injected intravascularly. The purpose of the present paper is to discuss the occurrence of hemorrhagic reactions in tuberculous guinea pigs at the site of injection of certain irritants following intravascular injections of various injurious substances.

It is known that tuberculous animals respond to various injurious agents with hemorrhage or with hemorrhagic necrosis. Tuberculin injected into the skin causes hemorrhagic necrosis in some guinea pigs, particularly when tuberculosis is advanced and the animals are highly sensitive to tuberculin. Hemorrhages occur in organs containing tubercles when tuberculin is introduced in the subcutaneous tissue or the peritoneal cavity. Tuberculin injected into the skin may produce hemorrhagic necrosis not only in tuberculous guinea pigs, but also in guinea pigs sensitized by large amounts of heat-killed tubercle bacilli (2). In such guinea pigs the tuberculin reaction is characterized by an extensive area of black necrosis and relatively slight edema. Tuberculous guinea pigs respond to various injurious substances not related to the tubercle bacillus, with hemorrhage

or hemorrhagic necrosis more readily than do non-tuberculous guinea pigs. The site of hemorrhage may be the skin, that is free of tubercles, or the organs (lung, spleen, liver) containing tubercles.

Dienes (3) made interesting observations on the occurrence of hemorrhages in skin tests on tuberculous guinea pigs. He studied the systemic reaction to egg white in tuberculous guinea pigs sensitized to egg white. He injected from 10 to 20 mg. of slightly virulent tubercle bacilli into the peritoneal cavities of guinea pigs. From 3 to 8 days later, he injected 0.1 to 1 mg. of egg white (dry weight) into the same site. When from 9 to 10 days after the injection, the guinea pigs were again injected with a larger amount of egg white (10 to 30 mg. dry weight) they died from 4 to 12 hours after the injection, with symptoms resembling tuberculin death. At autopsy, he found hemorrhages in tissues containing tubercles. If the guinea pig lived longer, or survived the shock, he observed bluish or purple discoloration or hemorrhages in the skin at the site of the reaction to egg white and also at the sites of recently healed wounds of operations. In several tuberculous guinea pigs, sensitized to egg white, the site of a tuberculin reaction became hemorrhagic after the injection of egg white, but he could not reproduce this observation in two subsequent experiments.

Paul Bordet (4) studied the effect of B. coli upon guinea pigs infected with a non-virulent strain of tubercle bacilli, the Calmette-Guérin bacillus. He injected 5 mg. of B.C.G. into the peritoneal cavities of guinea pigs. 3 weeks later, after the mesenteric lymph nodes had developed tubercles, 10 cc., that is, a large amount of a broth culture of colon bacilli, were introduced into the peritoneal cavities of guinea pigs. Some of the guinea pigs died within 1 day after the injection. Hemorrhages were found in the enlarged mesenteric lymph nodes containing tubercles.

Not only antigens but simple chemical compounds can produce shock symptoms and hemorrhages in tuberculous animals. For instance, an amount of sanocrysin harmless to non-tuberculous animals may kill tuberculous ones, and cause intense congestion and hemorrhages in organs that contain tubercles (5).

# Effect of the Injection of Bacterial Toxins upon the Tuberculin Reaction in the Guinea Pig

Groups of guinea pigs were infected with either of the following strains of tubercle bacilli: (1) bovine (Ravenel); (2) human (A<sub>1</sub> D), P<sub>2</sub>; (3) B.C.G. The amount of tubercle bacilli was varied: of the strain Ravenel, 0.01 mg.; of A<sub>1</sub> D, 2.5 mg.; of P<sub>2</sub>, 0.001 or 0.005 mg.; and of B.C.G., 5 mg. were used for infecting the guinea pigs. The guinea pigs were skin tested with old tuberculin from 6 weeks to 6 months after the infection. The old tuberculin was injected into the skin in dilutions of from 1:15 to 1:500. The bacterial filtrates, potent in the Shwartzman phenomenon, were injected from 18 to 48 hours after the injection of old tuberculin either intracardially or intravenously, or intraperitoneally, or subcutaneously. The intracardial or intravenous injections were made under light ether anesthesia. The bacterial filtrates were prepared by growing the culture on infusion agar in

Kolle flasks. The agar was seeded with 3 to 4 cc. (diluted 1:4 with saline immediately before planting) of a 20 hour plain broth culture. The growth was washed off with 14 to 16 cc. of normal saline solution containing 0.4 per cent phenol. The washings were pooled and centrifuged within 2 hours. The supernatant fluid was filtered through Berkefeld N or a Seitz filter. (See also the description of procedure by Shwartzman (6).) Bouillon cultures of B. coli, incubated for 6 to 10 days and filtered through a Seitz filter, were found potent and used in some of the experiments.

The following strains were used for preparing bacterial filtrates: (1) B. typhosus T.L., (2) B. coli, both obtained from Dr. Shwartzman, and (3) a strain of B. coli

TABLE I

The Hemorrhage-Producing Effect of an Injection of Typhoid Toxin upon the

Tuberculin Reaction in Guinea Pigs

No. of guinea pigs employed	Amount of typhoid toxin	Route of injection	No. of guinea pigs in which hemorrhage			
Стрюуса			Occurred	Did not occur		
	cc.					
2	0.25	Intracardial	0	2		
5	0.50	"	3	2		
5	1.00	"	4	1		
4	1.00	Intraperitoneal	2	2		
2	2.00	u	2	0		
3	2.00	Subcutaneous	1	2		
1	3.00	"	1	0		
1	4.00	66	1	0		
1	5.00	"	1	0		

isolated at New York Hospital. We have also used a filtrate of meningococci received from Dr. Shwartzman. The amount of filtrates injected and the route of injection are stated in Table I.

As shown in Table I, when guinea pigs infected with bovine or human virulent tubercle bacilli were injected into the skin with various dilutions of old tuberculin (from 1:15 to 1:500) and the skin tests followed from 1 to 2 days later by an intravascular, intraperitoneal, or subcutaneous injection of a filtrate from B. typhosus, the positive tuberculin tests became hemorrhagic about 4 hours after the injection of the filtrate.

As a rule general shock symptoms were present in guinea pigs injected with 0.5 cc. or more of the filtrate. Almost immediately after the injection of the

filtrate, the guinea pig's hair was ruffled and its breathing became dyspneic Some guinea pigs lay quietly on their sides, others had spastic movements. ation and defecation were frequent. Scratching was sometimes noticed. symptoms simulate non-lethal anaphylactic shock. Some of the guinea pigs died from 3 to 18 hours after the injection of filtrate. At autopsy hemorrhages were found in the organs containing tubercles and exudate in the pleural and peritoneal The changes found were not distinguishable from those in tuberculin shock.

The extent of the hemorrhagic reaction was proportional to the severity of the tuberculin reaction. This relationship could be best observed by comparing reactions in the same guinea pig, simultaneously injected with different dilutions of tuberculin. This is illustrated by the following protocol.

A guinea pig weighing 450 gm., which had been infected subcutaneously 7 weeks previously, was injected intracutaneously with 0.1 cc. of 1:45, 1:135, and 1:405 dilutions of old tuberculin. 2 days later the reactions at the site of the injections were as follows: Test 1 (1:45), light redness and edema 10 x 10 mm. raised 2 mm.; Test 2 (1:135), pink discoloration and edema 5 x 4 mm. raised 1 mm.; Test 3, doubtful. Immediately after the reading of the skin test 1 cc. of typhoid filtrate was injected intracardially. 1 hour after the injection the discoloration was dull red and 3 hours later the skin reactions were: Test 1 (1:45), mottled dark purple area 7 x 7 mm., edema 10 x 10 mm., raised 2 mm.; Test 2 (1:135), dark purple area 4 x 3 mm., edema 5 x 4 mm., raised 1 mm.; Test 3, doubtful. A few dark purple spots about 1 x 1 mm. were seen around Skin Tests 1 and 2. 1 day later the purple areas described above appeared to be necrotic.

In some guinea pigs not included in Table I, dilutions of old tuberculin from 1:400 to 1:1,200 caused only a slight reaction, i.e. a slight redness and edema, which did not change after the injection of bacterial filtrate, although the same animals showed hemorrhagic reactions at the sites of the more intense tuberculin reactions produced by higher concentrations of tuberculin. However, no significant correlation was found between the severity of the tuberculin reaction and the hemorrhagic reaction, when they were compared in different guinea pigs.

We have found some correlation between the appearance of general shock symptoms and the purple discoloration. Guinea pigs injected with amounts of filtrate which did not produce shock symptoms as a rule did not show the hemorrhagic reaction.

In occasional guinea pigs, a purple discoloration appeared at the site of the positive tuberculin reaction without the injection of bacterial filtrates. If such guinea pigs were injected with the filtrate the area of purple discoloration became more extensive.

In experiments not included in Table I, filtrates from B. coli and meningococci were used with results almost identical with those in Table I.

The question naturally arises whether tuberculin prepares the skin by itself or by the reaction it produces in the tuberculous guinea pigs.

In four normal guinea pigs, the hemorrhagic reaction failed to occur even when low dilutions of tuberculin (from 1:2 to 1:10) were employed. In the tuberculous guinea pigs, hemorrhages were observed at the sites of injections of tuberculin that showed definite tuberculin reactions but hemorrhages did not appear at the skin sites that were injected with dilutions too high to produce the tuberculin reaction.

These observations show that the inflammation produced in the hypersensitive animal, and not the tuberculin *per se*, is responsible for its preparatory action.

#### The Effect of the Injection of Bacterial Products upon the Tuberculin Reaction in Rabbits

It is well known that the tuberculous rabbit like the guinea pig is hypersensitive to old tuberculin injected parenterally. Both tuberculous rabbits and guinea pigs die with pleural and peritoneal exudation and hemorrhages in organs containing tubercles after an injection of an amount of old tuberculin apparently harmless to normal animals. However, there is a sharp contrast between tuberculous rabbits and guinea pigs in regard to the skin test with tuberculin. While the skin of tuberculous guinea pigs reacts with inflammation to small amounts of tuberculin, and the skin test is used as a delicate test to detect tuberculosis in guinea pigs, many tuberculous rabbits do not react at all to tuberculin injected into the skin and some of them react slightly. The reaction in the rabbit is very different from that in the guinea pig or other animals. It is characterized by redness and slight edema. Purple discoloration or necrosis does not occur. It was thought that examination of the tuberculin reaction in the rabbit, as a skin preparatory factor, might throw some additional light on the nature of the hemorrhagic reactions.

Rabbits infected intravenously with bovine tubercle bacilli were injected with 0.2 cc. of a fivefold dilution of old tuberculin. On the following day they received an intravenous injection of 0.5 cc. of typhoid filtrate, an amount sufficient to produce the hemorrhagic phenomenon in rabbits when the skin is prepared with filtrates from typhoid or colon bacilli or meningococci. The hemorrhagic reaction did not occur in any of eight rabbits so tested. The failure of the tuberculin reaction to prepare the skin is significant, for the rabbit is very susceptible to the hemorrhagic reaction; and in the rabbit, hemorrhages in the organs containing tubercles occur very regularly after the parenteral injection of old tuberculin.

These experiments indicate that tuberculin does not elicit a necrotic skin reaction in the rabbit and does not act as a preparatory agent for the hemorrhagic reaction.

Effect of an Intravascular Injection of Typhoid Toxins upon the Arthus Phenomenon in Tuberculous Guinea Pigs

Since the specific inflammation caused by tuberculin that prepares the skin of guinea pigs for the hemorrhagic reaction acts only in tuberculous guinea pigs, it seemed desirable to examine the action of horse serum in tuberculous guinea pigs sensitized to horse serum.

Dienes and Schonheit (7) demonstrated that tuberculosis modifies the hypersensitiveness to egg white and horse serum. When guinea pigs are first infected with tubercle bacilli, and then injected repeatedly with egg white, they react to egg white injected into the skin, not only with redness and swelling (like non-infected guinea pigs sensitized to horse serum), but also with a tuberculin type of reaction; *i.e.*, redness and swelling followed by necrosis. Sometimes the necrosis is preceded by a purple discoloration.

In order to study the Arthus phenomenon modified by tuberculosis as a skin preparatory factor in the guinea pig, animals were injected either with the Calmette-Guérin bacillus or with bovine strain Ravenel and sensitized with horse serum.

Tuberculous guinea pigs infected with B.C.G. and sensitized to horse serum, reacted with a necrotic type of inflammation to 0.1 cc. and without necrosis to 0.01 cc., of horse serum. When the guinea pigs so treated were injected with typhoid filtrate, the reactions became hemorrhagic or necrotic in all of the guinea pigs (Table II). The protocol of one typical experiment follows: Guinea Pig 129 was injected with 5 mg. of B.C.G. subcutaneously into the left groin. 1 week later, and twice at intervals of 3 days, 0.1 cc. of horse serum was injected also into the subcutaneous tissue of the left groin. 30 days after the infection, 0.1 cc. of horse serum was injected into the skin. The following day, at the site of the

injection, redness, edema  $45 \times 40 \times 3$  mm., and necrosis  $8 \times 8$  mm., were observed. 33 days after the infection, 0.01 cc. of horse serum was injected intracutaneously. The following day, light redness and edema  $40 \times 40 \times 4$  mm. were observed. On this day 1 cc. of typhoid filtrate was injected into the heart. 4 hours later purple discoloration appeared in an area of  $18 \times 12$  mm., and 20 hours later purple discoloration in an area of  $28 \times 25$  mm. and necrosis in an area of  $6 \times 2$  mm. was observed.

When we compare the Arthus phenomenon in non-tuberculous<sup>1</sup> and tuberculous (B.C.G.) animals sensitized to horse serum and the

TABLE II .

Arthus Phenomenon as Preparatory Agent in Tuberculous Guinea Pigs

Guinea pig No.		before se horse se	ensitization rum	Necrosis at the site of reaction to 0.1 cc. of horse serum	Hemorrhage at the site of reaction to 0.01 cc. of horse serum after intracardial injection of typhoid toxin	Systemic reaction
1	With	B.C.G.	,	Present	Present	
2	"	"		"	"	
3	"	**		**	"	
4	"	et.		"	"	,
5	With	strain	Ravenel	Absent		Died in 18 hrs.
6	"	**	"	"	"	""18"
7	"	"	"	Present	"	
8	"	"	"	Absent	"	""18"
9	"	**	"	Present	"	10
10	"	**	æ	"	"	
11	***	"	cc .	Present (slight)	Absent	
12	"	e e	"	Present	Present	" " 18 "

effect of bacterial filtrate upon the Arthus phenomenon in tuberculous and non-tuberculous guinea pigs, we find the following differences. In the non-tuberculous animals the Arthus phenomenon elicited by 0.1 cc. of horse serum was characterized by redness and swelling, in tuberculous guinea pigs by redness, swelling, and necrosis; the necrosis was preceded in some instances by purple discoloration. When 0.01 cc. of horse serum was used for testing the guinea pigs no purple discoloration or necrosis occurred in any of the animals. While the

<sup>&</sup>lt;sup>1</sup> See preceding paper.

intravascular injection of bacterial filtrate had no effect on the Arthus reaction in the non-tuberculous guinea pigs, it caused in the tuberculous guinea pigs hemorrhage or necrosis in the skin at the site of the injection of diluted horse serum.

In nine guinea pigs a virulent bovine strain was employed instead of B.C.G. and the infecting dose (subcutaneous) was reduced from 5 mg. to 0.01 mg.

Only five of the nine guinea pigs reacted with necrosis and one with hemorrhage to 0.1 cc. of horse serum, a smaller proportion than that in the guinea pigs infected with B.C.G. Hemorrhage appeared in the skin at the site of the injection of 0.01 cc. of horse serum, in six of the seven guinea pigs, after the intracardial injec-

There seems to be a relationship between the capacity of guinea pigs to react with necrosis to 0.1 cc. of horse serum and the capacity to react after intravenous injection of bacterial filtrate with hemorrhage at the site of former injection of 0.01 cc. of horse serum. As a rule, the inflammation produced by horse serum acted as a preparatory factor only in those guinea pigs which reacted with necrosis to 0.1 cc. of horse serum.

Effect of an Intravascular Injection of Typhoid Toxin upon Skin Lesions Produced by Silver Nitrate in Tuberculous Guinea Pigs

In contrast to the result in normal animals, hemorrhagic reactions appeared in eight of ten tuberculous guinea pigs at the site of the injection of silver nitrate after the injection of bacterial filtrate (Table The area of hemorrhage was, as a rule, considerably more extensive in the tuberculous than in the non-tuberculous guinea pigs described in the preceding paper. The question arises whether the observed difference in normal and tuberculous guinea pigs is due to a difference in the reaction of the skin to silver nitrate itself, or to their reaction to the bacterial filtrate. There was no difference noted in the reaction of the skin to silver nitrate injections in tuberculous and in non-tuberculous guinea pigs. However, tuberculous guinea pigs react differently from normal ones to bacterial filtrates. They die from a relatively small amount of filtrate with hemorrhages in the tissues containing tubercles and effusions in the pleural and peritoneal cavities.

TABLE III
Silver Nitrate as Skin Preparatory Agent in Tuberculous Guinea Pigs

Guinea pig No.	Preparatory agent, 0.1 cc. silver nitrate	Injury-producing agent, typhoid toxin	Hemorrhagic reaction	Systemic reaction
1	1:40 1:200	α. 2.5	Present	
	1:1,000		riesent	
2	1:40 1:200	2.0		
	1:1,000	2.0	et.	Died in 24 hrs.
3*	1:100			
3.	1:200 1:1,000	2.0	Absent	
	1:100			
4	1:200 1:1,000	1.5	Present	" " 24 "
ļ	1:100			
5	1:200 1:1,000	1.5	"	" " 24 "
	1:100	ľ	•	~1
6	1:200	1.5	•	" " " " "
	1:1,000		j	" " 24 "
7	1:100 1:200	1		
	1:1,000	1	**	" " 24 "
8	1:20	1	"	""24"
9	1:100		"	24
	1:200 1:1,000	1	" Absent	
	1:100		- TOSCIIE	
10	1:200	1	a l	

<sup>\*</sup> Infected with 0.000,001 mg. human tubercle bacilli 3 weeks before experiment.

This difference between the behavior of the tuberculous and non-tuberculous guinea pigs toward the bacterial filtrate, the injury-producing factor, may explain why the same irritant, silver nitrate, acts as a preparatory factor in the tuberculous and often fails to act in the normal guinea pig.

## Tuberculin as Injury-Producing Factor

As described above, the parenteral injections of filtrates from B. typhosus, B. coli, and meningococci were active in tuberculous guinea pigs as injury-producing agents. Since these filtrates produce anaphylactoid symptoms in the non-tuberculous, and shock not distinguishable from the tuberculin shock in the tuberculous guinea pig, the question arises whether tuberculin would act as an injury-producing factor. Tuberculin is considered non-toxic to normal guinea pigs, since 5 cc. injected intraperitoncally or intravascularly does not produce obvious symptoms. The lethal dose for tuberculous animals is about 0.25 cc.

Seven guinea pigs were infected subcutaneously with from 0.001 to 0.01 mg. of human tubercle bacilli and tested from 6 weeks to 3 months after infection (Table The tuberculin reactions were well defined by redness and edema. was no purple discoloration or necrosis before the intraperitoneal injection of tuberculin, except in two guinea pigs (Nos. 6 and 7 in Table IV). In these guinea pigs, the extent of hemorrhage was increased after injection of bacterial filtrate.

Table IV shows that tuberculin acted as an injury-producing agent in all guinea pigs that died subsequently from tuberculin shock. Hemorrhage failed to occur in one of two animals that survived the intraperitoneal injections of tuberculin.

# Effect of Intravascular Injection of Witte Peptone or Soluble Starch upon

Since the hemorrhagic reactions were produced by parenteral injections of the products of various bacteria not related to tuberculosis, and systemic reactions, such as dyspnea, tremor, ruffling of the hair, urination and defecation, spastic movements in some of the guinea pigs, i.e. symptoms of stimulation of smooth muscles, were constant, an attempt was made to ascertain whether toxic bacterial products

could be replaced by non-bacterial substances which cause similar anaphylactoid symptoms in guinea pigs. For this purpose we have used Witte peptone (8) and soluble starch (9).

TABLE IV

The Hemorrhage-Producing Effect of Intraperitoneal Injections of Tuberculin upon

Skin Reactions to Tuberculin

Guinea pig No.	Preparatory agent, tuberculin injected into the skin	Injury-producing agent, tuberculin injected into the peritoneal cavity	Hemorrhagic reaction	Systemic reaction
		cc.		
1	1:25 1:100 1:400	2	Present	Died with tuberculin shock
	1:25	}	"	
2	1:25		Absent	" "
2	1:100	1.5	Absent	
	1:400			
	1:25		Present	
3	1:100	1.25	Absent	
3	1:400	1.25	Absent "	
	11.700	j		,
	1:25		<u> </u>	
4	1:100	1	Present	и и
	1:400	}		
	ł	1	1	
	1:25	ļ		Hair ruffled, dyspnea:
5	1:100	0.6	Absent	Hair ruffled, dyspnea;
	1:400		]	Salvived
	1:25*	1		
6	1:100	0.5	7	Died with tuberculin
U	1:400	0.5	Present	shock
	1.400	1	1	
	1:25*	ł	1	
7	1:100	0.3	Absent	Hair ruffled, dyspnea;
	1:400*	}	}	scratching; survived
	<u> </u>		<u>!</u>	Į.

<sup>\*</sup> Small areas of purple discoloration which increased after the intraperitoneal injection of tuberculin.

In the experiments with Witte peptone twelve guinea pigs injected with virulent tubercle bacilli and five guinea pigs infected with B.C.G. were employed. In the guinea pigs of Table V the reactions to tuberculin, diluted 1:50, 1:200, or

TABLE V  Guinea pig No.  Preparatory agent, 0.1 cc. tuberculin injected into skin  Injury-producing agent, Witte agent, Witte injection  Preparatory agent, 0.1 cc. tuberculin injected into skin  Injury-producing agent, Witte agent, Witte injection
TABLE V
Guinea pig No. Preparatory Preparatory Preparatory
agent, 0.1 cc. Injury no location upon the Type
Jected into skin agent, Witte after the property of the period of the pe
Peptone Skin Peptone Systemic Post National S
1   1:50   sm.   Systemic reaction
1:200
$1:1,000$ $0.2$ $P_{resent}$
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
1:200
1:1,000   0.2   Present*   Presen
3   1:50     Died with
3   1:50   Died within 18 hrs.
$\left \begin{array}{cc} 1:1,000 \end{array}\right   0.2  \left \begin{array}{cc} \end{array}\right $
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$
1:2,000   0.1   Present*
5   1:50   " Dicd in a
1 -00 1
1:2,000   0.1   " *
6 1:50 / " * /
1:200
$\int \frac{1:2,000}{}$ 0.1 $\int {}$ "
7   1:50
1:200
$\left \begin{array}{cccccccccccccccccccccccccccccccccccc$
8 $1:50$ $Absent$
1:200
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$
1.30
$ \begin{array}{c c}  & 1:200 \\ \hline  & 1.5 \end{array} $
10
1:1,000   0.05   Present*
$\begin{pmatrix} 1 & 1:50 & & & & & & & & & & & & & & & & & & &$
1:1,000   0.05   " *
1:50
1:30 1:200   0.05   Absent
Small and Present*
creased definition of hemorrhage.

A very small area of hemorrhage was present before the injection of peptone:

1:1,000, were well defined by redness and edema. In seven guinea pigs there was a purple discoloration at the site of the reactions to a 1:50 or 1:200 dilution of tuberculin. Within 5 hours after the injection of peptone, the hemorrhagic areas increased in size. Hemorrhage occurred after the injection of peptone in all animals except one. The occurrence of hemorrhage was more frequent with dilutions of tuberculin that produced intense inflammation. In some of the guinea pigs scratching, dyspnea, spastic movements, and urination were observed within a few minutes after the injection of peptone. One guinea pig died in 5 minutes

TABLE VI

The Effect of Intravascular Injection of Soluble Starch upon the Tuberculin Reaction

Guinea pig No.	Preparatory agent, 0.1 cc. tuberculin injected into the skin	Injury-producing agent, 10 per cent soluble starch	Hemorrhage after the injection of soluble starch	
1	1:50 1:200 1:1,000	и. 10	Present* " Absent	
2	1:50 1:200	10	Present Absent	
3	1:50 1:200	10	Present Absent	
4	1:200	10	Present	
5	1:50 1:200	10	ec ±	

<sup>\*</sup> A very small area of hemorrhage was present before the injection of starch; its size increased definitely after the injection of starch.

and four in from 6 to 18 hours after the injection of peptone. At autopsy no hemorrhages were found in the organs. This is unlike the findings after the injection of typhoid or *B. coli* toxins.

When a similar experiment was made with five guinea pigs infected with B.C.G., only one of them reacted with increase of hemorrhage at the site of tuberculin reaction, after the injection of peptone.

In the experiments with starch five tuberculous guinea pigs were injected with various dilutions of tuberculin and later injected intracardially with 10 cc. of soluble starch. The starch produced no obvious symptoms except dyspnea. Table VI shows that after the injection of starch, hemorrhage either increased or

developed in all of the guinea pigs at the site of the more intense tuberculin

In the previous paper (1) it was suggested that there is a relationship between the capacity of irritants to elicit hemorrhage or hemorrhagic necrosis in the skin of susceptible guinea pigs, and their capacity to act as preparatory agents for the Shwartzman phenomenon. ments reported in the present study show that in tuberculous guinea pigs tuberculin, horse serum (in animals sensitized to it), and silver nitrate act regularly as skin preparatory agents. The experior B. typhosus, concentrated broth, or turpentine (each of these materials was employed in four to six guinea pigs) failed to prepare the Toxins from B. coli skin for the hemorrhagic reaction. Tuberculin, horse serum, and silver nitrate caused hemorrhages in the skin of some tuberculous guinea pigs, while the latter group of substances did not do so. observations in tuberculous and non-tuberculous guinea pigs, therefore, are in agreement in relation to the capacity of irritants to cause hemorrhages in susceptible animals and to act as preparatory agents.

It seems significant that, on the one hand, 0.01 cc. of horse serum producing mild inflammation acted as a preparatory factor in tuberculous guinea pigs that reacted with necrosis to 0.1 cc. of horse serum; on the other hand, 0.1 cc. of horse serum was inactive as a preparatory agent in tuberculous and non-tuberculous guinea pigs that reacted to it with redness and edema, but with no necrosis.

Intravascular injections of typhoid toxin caused hemorrhages at the site of injection of silver nitrate more frequently in tuberculous than in non-tuberculous guinea pigs. more extensive in tuberculous animals. The question arises whether The area of hemorrhage was usually the observed difference in normal and tuberculous guinea pigs is due to a difference in the reaction of the skin to silver nitrate itself, or to their reaction to the bacterial filtrate. There was no difference noted in the reaction of the skin to silver nitrate injections in tuberculous and non-tuberculous guinea pigs. However, tuberculous guinea pigs react differently from normal ones to bacterial filtrates. relatively small amount of filtrate with hemorrhages in the tissues containing tubercles and effusions in the pleural and peritoneal cavities. They die from a

This difference between the behavior of the tuberculous and non-tuberculous guinea pigs toward the bacterial filtrate, the injury-producing factor, may explain why the same irritant, silver nitrate, acts as a preparatory factor in the tuberculous, and fails to act in the normal guinea pig.

As injury-producing agents, bacterial toxins from B. typhosus, B. coli, and meningococci were used with consistent success. Of the three kinds of filtrates, that from meningococci was the most potent. The injury-producing substance can be effectively introduced by the intraperitoneal and subcutaneous routes.

Since the production of hemorrhage by bacterial filtrates and by tuberculin was associated with shock symptoms, peptone and starch, which produce symptoms of anaphylactoid shock in the guinea pig, were examined as injury-producing factors. Both Witte peptone and starch were effective in sublethal doses as injury-producing factors. These observations show that substances of non-bacterial origin that cause anaphylactoid shock symptoms may produce hemorrhages in the skin at the site of the tuberculin reaction.

After the introduction of injury-producing agents, namely B. coli, typhoid, or meningococcus toxins, tuberculin or peptone, a considerable number of the guinea pigs died. Large amounts of these substances, as might be expected, caused death more frequently than small amounts. Regardless of the amount of injury-producing substance injected, the animals that died as a rule had hemorrhages in the skin lesions. It appears therefore that the hemorrhage-producing effect of the substances mentioned above is related more closely to the systemic reaction than the amounts injected.

#### SUMMARY AND CONCLUSIONS

- 1. When toxic filtrates from cultures of B. coli, B. typhosus, or meningococci are injected into the blood stream, peritoneal cavity, or subcutaneous tissue of tuberculous guinea pigs, the skin at the site of a tuberculin reaction becomes hemorrhagic. The extent of the hemorrhage is proportional to the severity of the tuberculin reaction demonstrable by tests with various dilutions of tuberculin.
- 2. Tuberculin does not prepare the skin of non-tuberculous guinea pigs for this hemorrhagic reaction.

- 3. Tuberculin does not produce an intense or necrotic inflammation in the skin of tuberculous rabbits and fails to prepare the skin for the hemorrhagic reaction.
- 4. Tuberculin injected into the peritoneal cavities of tuberculous guinea pigs causes a hemorrhage in the skin at the site of a tuberculin reaction.
- 5. All guinea pigs infected with B.C.G., and most of those infected with a virulent strain of tubercle bacilli, when sensitized to horse serum and injected intracutaneously with 0.1 cc. of horse serum, react with redness, edema, and necrosis; and in some instances the necrosis is preceded by hemorrhage. When horse serum is injected into the skin of these guinea pigs in such dilution that only redness and edema result, the subsequent intravascular injection of typhoid filtrate produces hemorrhage at the site of reaction regularly in those infected with B.C.G. and frequently in those infected with a virulent strain.
- 6. Filtrates from B. coli, B. typhosus, or meningococci injected into the skin of tuberculous guinea pigs do not produce visible inflammation. When these injections are followed by intravascular injections of the same material hemorrhages do not occur in the skin.
- 7. When concentrated broth or turpentine is introduced into the skin of tuberculous guinea pigs and later typhoid filtrate is injected into the vascular system, hemorrhages do not occur in the skin at the site of inflammation.
- 8. The majority of guinea pigs that receive an intravascular injection of typhoid filtrate react with hemorrhage at the site of the injection of the silver nitrate. The incidence of hemorrhagic reaction in tuberculous guinea pigs is higher than in non-tuberculous guinea pigs that received similar injections of silver nitrate and typhoid toxin.
- 9. In tuberculous guinea pigs the skin can be prepared for the hemorrhagic reaction not only by bacterial toxins but also by tuberculin, horse serum, and an inorganic chemical, silver nitrate.
- 10. In the guinea pig the skin preparatory agents, *i.e.* tuberculin in the tuberculous guinea pig, diphtheria toxin and silver nitrate in both tuberculous and non-tuberculous guinea pigs, tend to produce hemorrhages in the skin even without subsequent injection of a toxic bacterial product. This property of the skin preparatory agents may be essential in their action.

11. Hemorrhages occur in the skin at the site of tuberculin reaction not only after the intravascular injection of bacterial toxins or tuberculin, but also after the injection of substances of non-bacterial origin; namely, peptone or soluble starch.

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### COMPARISON OF THE HEMACYTOLOGICAL CONSTITUTION OF MALE AND FEMALE RABBITS

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Previous reports from this laboratory on the hemacytological constitution of the rabbit have been limited to observations on male animals. These studies have now been extended to normal females and to females presenting various defects referable to constitutional (hereditary) or environmental influences. It is the purpose of the present paper to present one aspect of these studies; namely, a comparison of the blood cytology of normal male and female litter mates. These comparisons were made in order to determine whether there were statistically significant differences between values obtained for any of the blood cells of the two sexes. Furthermore, correlation coefficients were calculated with the purpose of disclosing to what extent a knowledge of the blood cytology of a buck was relevant information concerning the blood cytology of a litter mate sister.

#### Material and Methods

Observations were made on the blood cytology of 41 pairs of normal rabbits, each pair consisting of a buck and a doe of the same litter; an analysis of these observations forms the basis of the present report.

Animal Material.—All animals were bred and raised in this laboratory, and all were bred in pure line from standard stock with the exception of three pairs of Rexes. These were obtained by a back-cross mating of an  $F_1$  Rex to a pure line Rex parent and were of normal Rex type. The breeds and the number of animals in each breed were as follows: Havana 7; Dutch and Rex 6; Himalayan and Beveren 4; English, Chinchilla, and Belgian 3; Polish and Silver 2; Tan 1. All the rabbits were virgin stock and gave no evidence of disease as determined by careful clinical examination. The age ranged from a minimum of 16.0 weeks to a maximum of 37.8 weeks, with a mean value of  $26.0 \pm 0.59$  weeks.

Housing and Feeding.-The buck and doe comprising a pair were weaned at

the same time and housed in separate cages in well ventilated rooms receiving sunlight. The uniform diet fed to all animals consisted of a commercial food supplemented with hay, oats, and a free supply of water.

Blood Examinations.—The technique of the blood examinations was, with minor modifications, that employed in this laboratory for several years. red cell and white cell counts were made with automatic, standardized pipettes, the hemoglobin estimations were made with a Newcomer hemoglobinometer, and platelet counts were determined by the method of Casev and Helmer (1). Two white blood pipettes were employed at each bleeding. For the differential white cell examinations the supravital, neutral red technique was followed and 300 cells were counted at each determination. When two observers examined the smears, each counted 150 cells on two different preparations, and when three individuals conducted the examinations, two counted 100 cells on two different smears and the third counted 50 cells on each of the same two preparations. All of the total white and red cell counts and the hemoglobin determinations were made by a single Three complete blood examinations were conducted on each animal, and with five exceptions these were made on three successive mornings; for the five pairs of animals, the spread of the three examinations was over a period of 5 days. Twenty-two pairs were counted from October 14 to November 5, 1932, and the remaining nineteen pairs were examined during the period from October 3 to October 26, 1933. All the bleedings took place in the morning, and whenever a buck was bled, its litter mate sister was bled, either immediately before or immediately after, the interval between the withdrawal of blood from the buck and its sister in no case exceeding 10 minutes.

Analysis of Material.—Mean blood cell values were calculated for each animal by averaging all the counts on the particular animal. From these mean values the mean and standard error of the mean were determined for each blood constituent for the 41 males and the 41 females. Since the purpose of these observations was to compare the blood cytology of litter mates of different sexes, the difference between the values for each pair of animals was determined for each of the fourteen blood constituents under consideration. The mean difference

 $(M_d)$  was obtained for each blood factor by the formula  $\frac{\Sigma_d}{n}$  and the standard error of the mean difference from the formula

$$\sqrt{\frac{\sum_{(d)^2}-\sum_d M_d}{n(n-1)}},$$

d representing the difference between the numerical observations on any pair of animals. This method of deriving the standard error of the difference eliminates the variation due to breed; that breed exerts a considerable influence on the blood cytology has already been shown (2). A mean difference was considered significant when it was at least  $2\frac{1}{2}$  times its standard error (t = 2.5 or 2.5 +); that is,

the probability of such a difference occurring by chance is 1 or less than 1 in 100 (P = 0.01 or 0.01 -). Simple zero order correlations  $(r_{12})$ , and first order partial correlations eliminating age  $(r_{12\cdot 2})$ , were then calculated by the usual statistical procedures. For determining the significance of these correlation coefficients, the formula

$$t = \frac{r}{\sqrt{1 - r^2}} \cdot \sqrt{n' - 2}$$

was used. Published tables (3) are available for translating various levels of t into terms of probability. When the probability of a correlation coefficient occurring by chance was 1 or less than 1 in 100 (P = 0.01 or 0.01-), the correlation was taken to be significant; when the probability of chance influence was between 1 and 5 in 100 (P = 0.01 + to 0.05), the result was interpreted as probably significant. A last calculation involved the determination of regression equations. In each case the equation represents the regression of the male on the female value.

#### RESULTS

The mean blood cell level of each of the 82 animals under consideration is shown in Table I. The mean values for the male and female groups, together with the mean difference for each factor, are presented in Table II. Table III gives the zero order and first order correlations between the blood cell values of the litter mate pairs, and Table IV presents regression equations for each cell element. The correlation coefficients of Table III are presented graphically in Text-fig. 1.

It is seen from Table II that with the exception of the red cell count and hemoglobin content, no significant differences were observed between the two groups. The red cell count for males was higher than that for females by a mean difference of  $300,000 \pm 83,600$ . This difference is significant since it is 3.6 times its standard error. The hemoglobin value for the male group was higher than that observed in the female group by a mean difference of  $5.0 \pm 0.97$  per cent, which is 5.2 times its standard error, a significant value.

The simple zero order correlations between the blood cells of the bucks and their sisters (Table III, Text-fig. 1) show a significant positive relationship with respect to the following cells: Red blood cell count, white blood cell count, hemoglobin content, neutrophils and lymphocytes in absolute and relative numbers, and basophils and monocytes in per cent. No significant correlations were observed

1	1 1	1	The Blood	l Cytology of V	irgin Malc and Fem
No. Breed*	Litter No. Age	R	н	w	
		Q, ô	9 0	·	P N
1 HA	wks.	+0000 +0000	per cent per cent	9	9 8 9
2 "	$ \begin{array}{c cccc} 24 & 24.0 \\ 25 & 22.9 \end{array} $	549 545	71.6 65.8	1 200	+000 +000 +0 +
3 ("	25   22.9	603   559   543   556	77.3   66.4	820 837 866 738	541 938 437 41
4	20   31.0	661	72.9   72.2   74.5   69.0	681 631	483 460 412 35
6 "	26   23.4   26   23.6	528   480	74.5   69.0   57.3   60.4	586   927	711 604 258 490
7 "	20 20.0	552   458   6	59.3   64.3	662   451 715   647	606 468 413 250
8 D 0 0	55   29.0	107	74.5   74.8   1.1   61.2	800   735	613 875   506   351
10   "	06 1 6 - 1	632   536   8	$\begin{bmatrix} 1.1 & 61.2 \\ 0.4 & 74.5 \end{bmatrix}$	839   832   1	340 674   364   463 110 790   455   358
19 1 11 1	13   24.9   6	552   78	8.3 64.2		541 631 347 264
12 / "   1	$\begin{vmatrix} 20 & 23.9 & 5 \\ 20 & 23.9 & 5 \end{vmatrix}$	83   544   69	74.5	764   790   8	525 773 230 484 90 584 282 302
14   R   1	51   20.5   3	34   567   67	.1 70.7	481   489   6.	33 593 191 265
15 " 10	28.7 60	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	.7   60.1   7	$     \begin{array}{c cccccccccccccccccccccccccccccccc$	70 663 267 340
17 " 10	6 27.4 64	6 605 88.	5 74	60   732   79	
18 " 10	9   26.3   50	4   474   83.	7 76.6 83	09   610   68 38   825   58	3 683 252 233 4
20 H 111	24.7 56	1   532   86	$\frac{1}{1}   \frac{78.3}{78.3}   \frac{75}{75}$	55   692   864	1 646   555   500   4
21 " 53	1 00.0 1 ()	5   700   74.9	74.6 76	8   664   604	575   264   210   30
22 " 57	26.3 551	1 400 1 109 1	70.1   570		565   446   312   57
24   BA   31	334	468 68.8	040	689   587	591   260   263   44 540   205   272   31.
25 " 31	24.9 482	485   57.3 527   56.8	59.4 683	1 092	560 317 345 63.
27 " 21 37	37.6 525	527   56.8 613   66.3	57.8 800	612 402	491   270   320   39. 574   311   343   38.
28   C   26	24.1 545 32.6 498	503 74.5	68.7 922 61.5 799	789 739	548   515   319   55.3
29 " 43	25.7 520	504 68.3 510 63.6	63.8   1137	1 1024 1 - 1	702   250   391   30.9
31 B 93	24.9 572	510   63.6   562   69.1	61.2   1285 64.8   837	1089 604	540   333   350   29.6 536   479   535   37.3
32 " 155	$ \begin{array}{c cccc} 16.0 & 539 \\ 24.9 & 655 \end{array} $	444   72.5	60.6 723	848   490 7	00   269   313   31.9
34 E   169   39	21.4 564	510	59.2   683	538   584 6 643   568 50	59   264   216   36.8 02   282   199   42.0
35   "   46	31.6 548 27.1 572	504 70.1	61.5   410   58.1   619	629   312   68	33   132   290   31.9
36 " 101 37 P 20	25.4 549	525   66.2	64.1 613	855   610 59 807   411 60	1 =   *=0   =
38 " 20 44	24.0 615 27.3 580	532 77.7	71.9   558 59.7   786	742   486 54	
39   SA   14   1	20 2   45-	622   77.4   7	6.0 599	959   669 563 773   642 785	3   409   441   52.1 4
41   7   14	30.9   332	100		928   472   482	1 1 1
	20.9   555	577   ~~ -   -		921   600 =00	1 1 200   02.10
* HA = Havana; D	= Dutch; R	= Rex: H =	Himal	655   690   655	281   410   43.5   46
		-,	malayan;	BA = Bevere	n; C = Chinchilla; B

s. Mean Values for Male and Female Individuals

====	В	%		Е		E	ic	L		L	70	N	[	М	%	No.
ç	- d'	Ş	_ -	8	ç	o'	ç	o"	ç	o <sup>2</sup>	Ç	ð	Ş	ð	Ŷ	
+0			- -	+0	+0			+0	+0			+0	+0		-	
45	4.1	5	.2	9	8	1.1	0.9	256	277	31.9	33.0	85	97	10.6	11.7	1
53	9.8		.2	5	5	0.6	0.7	243	258	28.5	34.5	120	64	13.7	9.3	2
52	7.6		.1	7	4	1.0	1.2	192	189	28.3	30.0	95	56	14.1	8.8	3
46	7.7		.0	3	9	0.6	1.0	243	311	41.6	33.5	39	68 42	6.2 9.7	7.4 9.5	4 5
28	2.6		.3	4	10	0.6	2.1	162 157	121 150	24.9 21.9	26.9 23.6	67 39	86	5.5	12.8	6
50 37	0.9		.9	6	13	1.4	1.9	318	174	39.7	23.7	75	47	9.3	6.5	7
33	3.1		3.9	8	26	0.9	3.0	255	368	30.1	44.3	96	48	11.2	5.7	8
32	5.9		1.4	15	18	2.0	2.6	309	358	40.9	49.8	41	52	5.2	7.2	9
18	5.		2.2	25	15	3.8	1.9	328	219	49.2	27.6	41	53	6.6	6.6	10
53	3.3		5.9	18	16	2.3	2.0	389	378	50.7	47.6	51	41	6.5	5.2	11
21	5.		4.2	11	9	2.2	1.8	222	150	45.7	30.5	34	45	7.1	9.5	12
16	3.9		2.5	13	6	2.2	0.9	240	257	40.4	38.8	43	34	7.3	5.1	13
21	6.		2.8	6	16	0.8	2.1	260	277	33.0	36.2	89	55	11.2	7.4	14
92	2.		2.6	6	20 10	0.9	2.6	267 158	245 240	42.5 31.4	33.8 40.0	33	54 56	4.7 6.7	7.4 9.0	15 16
71 93	12. 5.		1.9	8	12	1.0	1.4	368	241	43.6	41.4	45	79	5.3	9.5	17
27	3.		3.9	3	5	0.4	0.8	397	235	52.0	33.9	31	37	4.2	5.5	18
17			2.6	5	8	0.5	1.2	468	375	53.8	56.4	104	54	11.8		19
43	ı		6.8	7	9	0.9	1.6	209	209	27.5	33.9	89	45	11.8	7.3	20
38			7.0		7	1.0	1.3	226	168	40.8	31.7	39	58	6.8	10.8	21
44			6.4	15	12	2.5	1.6	285	272	43.9	39.6	73	90	12.0	12.8	22
33	- F	.8	6.0		2 2	0.9	0.4	118	136	23.5	24.3	32	41	6.5	7.3	23
38		.0 .9	6.7 7.5	22 43	7	3.2	0.4	249 326	176	37.0 41.3	30.3	58 47	33	8.6 5.9		24 25
70		.8	9.0		39	0.7	4.8	283	282	31.0	35.9	102	81	11.2		26
. 49		4	5.7		6	0.3	0.8	429	329	54.0	38.1	72	88	9.2		27
3.	1 2	.8	3.3		17	1.2	1.7	662	587	57.9	56.9	97	42	8.5		28
4		.0	4.6		31	3.7		594	417	46.5	38.7	98	57	7.4		29
5		.9	7.2		19	1.8		492	418	59.1	49.0	37	38	4.3		
.   2		.4	3.9		13	2.1		322	247	44.3	45.6	67	42	9.4		
;   8 }   9			13.7 14.7		25 31	2.1	1	1		37.4 51.2	,	41	43	6.0		
		7.1	8.0		8	2.8				36.6		39 55	109	9.5		1
	1	5.0	6.9	1	4	0.8		1	7	29.1		41	99	6.8		
		5.9	6.0		15					49.3		46	96	8.2		
		1.9	2.5		13						44.0	63	40	8.0		1
		1 9	3.	4	5		1	1					48	3.7	1	38
	- 1	3 0	5.		19		i					1	81	14.1	1	
1		2.9	3 · 9.	1	31								163	15.3		1
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leleia	elgian: E = English: P = Polish: SA = Silver: T = Tan															

Belgian; E = English; P = Polish; SA = Silver; T = Tan.

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The Blood Cytology of Virgin Male and Pemale Litter Male Rabbits. Mean Values for Male and Pemale Litter Male Rabbits. Mean Values for Male and Pemale Groups $ \begin{array}{c ccccccccccccccccccccccccccccccccccc$
8 4 8 H H 1 8 H 1 8 Class Clas
ale and Female  3266 $\pm$ 142  3464 $\pm$ 124  -198 $\pm$ 159  M %  N %  8.4 $\pm$ 0.5  8.3 $\pm$ 0.5  6.1 $\pm$ 0.5  4.1; $N$ = absolution;  = relative count;
Male and Fema  N  3464 ± 142  -198 ± 159  M %  8.4 ± 0.5  8.3 ± 0.5  0.1 ± 0.5  "nt; $N$ = abso  phocyte coun; $N = 1$ = relative ec
$\begin{bmatrix} & Values for M_a \\ & N \% \\ & & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & \\ & & & & \\ & & & \\ & & & & \\ & &$
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abbits. Mean $ \begin{array}{c ccccccccccccccccccccccccccccccccccc$
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Rabbits. Meam  Rabbits. Meam  72.0 $\pm$ 1.5  67.0 $\pm$ 1.5  67.0 $\pm$ 1.6  1.0 $\pm$ 1.3  39.2 $\pm$ 1.6  36.4 $\pm$ 1.3  11 count; $H = hemo$ relative basophil
H 2
7 15   16   16   17   16   17   17   17   17
23.22   ####   50.22.23   ###   ###   E
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $
ale and Femal.  W  7221 ± 255  7412 ± 225  7412 ± 205  1.5 ± 0.2  1.8 ± 0.2
fale and Fema  W  7221 $\pm$ 23  7412 $\pm$ 23  7412 $\pm$ 23  1.5 $\pm$ 0.2  1.8 $\pm$ 0.2  cell count; P  tt; E = abso.  neutrophii co. $\%$ = relative
7221 = 7412 ± 191 ± 191 ± 191 ± 191 ± 191 ± 191 ± 191 ± 191 ± 100
% e. i. i. i. i. i. i. i. i. i. i. i. i. i.
100 100 100 1 1 1 1 1 1 1 1 1 1 1 1 1 1
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83 of Virgin 5642 ± 5642 ± 5342 ± 450 ± 32 462 ± 34 62 ± 43 1 = total whi basophil co 7 = relativ Cyte count;
108y of Vir 5042 5342 5342 7300 ± 462 ± 462 ± 700 ± 1000yte count
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between the platelet values, the absolute number of basophils and monocytes, and the absolute and relative number of eosinophils. With only one exception the significance of these correlations was not altered when first order partial correlations were calculated holding age constant. Eliminating the factor of age reduced the correlation

TABLE III

Zero and First Order Coefficients of Correlation between Blood Cell Values of 41 Male

and Female Litter Mate Rabbits

	Zero or	der coefficie	nt of correl	ation	First order coefficient of correlation— Age constant			
	712	σ <sub>712</sub>		P	$\sigma_{r_{12.3}}$ $\sigma_{r_{12.3}}$		t	P
R	+0.635	0.094	5.13	0.01-	+0.684	0.085	5.78	0.01-
W	+0.560	0.109	4.28	0.01-	+0.547	0.112	4.03	0.01-
P	+0.149	0.123	0.09		+0.156	0.156	1.03	<b>-</b>
н%	+0.712	0.080	6.33	0.01-	+0.712	0.079	6.25	0.01-
N	+0.306	0.143	2.00	0.05	+0.244	0.151	1.55	_
В	+0.119	0.156	0.75		+0.149	0.157	0.93	-
E	+0.211	0.151	1.35		+0.250	0.150	1.59	-
L	+0.693	0.082	6.00	0.01-	+0.699	0.082	6.02	0.01-
M	+0.233	0.150	1.49		+0.256	0.150	1.63	<b>-</b>
N %	+0.316	0.142	2.08	0.05-	+0.333	0.142	2.18	0.04
в %	+0.305	0.143	2.00	0.05	+0.313	0.144	2.03	0.05-
E %	+0.132	0.155	0.83		+0.132	0.157	0.82	_
L %	+0.461	0.125	3.24	0.01-	+0.459	0.126	3.18	0.01-
М %	+0.409	0.132	2.80	0.01-	+0.395	0.135	2.65	0.01-

R= total red cell count; W= total white cell count; P= platelet count; H= hemoglobin content; N= absolute neutrophil count; E= absolute basophil count; E= absolute eosinophil count; E= absolute lymphocyte count; E= absolute monocyte count; E= relative neutrophil count; E= relative basophil count; E= relative eosinophil count; E= relative lymphocyte count; E= relative monocyte count.

coefficient for neutrophils per cubic millimeter from a probably significant value to one of no statistical significance.

#### DISCUSSION

The experiments were planned with the purpose of eliminating so far as possible those variables which could be controlled. Thus the buck and doe comprising a pair were weaned at the same time and

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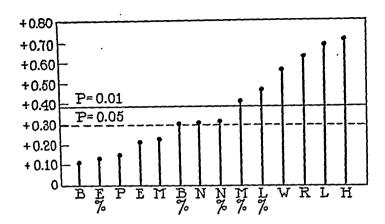
placed in separate cages where they were fed a similar diet. All hematological procedures were carried out at the same time and under the same conditions, so that the technical error was presumed to be uniform. Moreover, time of the day and season were constant factors since all counts were conducted in the morning during the 1 month period from October 3 to November 5 of 2 years. It would

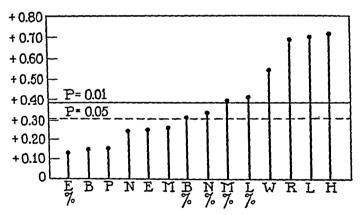
Linear Regression Equations Calculated from the Blood Cytology of Virgin Male and

	Row 1 . J. W. Ille Blood C
	Female Litter Mate Rabbits  Female Litter Mate Rabbits
R	Male Rabbits 83 of Figur Male and
W	
	$\overline{M} = 2,052,000 + 0.672 \text{ F}$ $\overline{M} = 2,626 + 0.672 \text{ F}$
P	$\frac{1}{2}$ , $\frac{1}{2}$ , $\frac{1}{2}$ , $\frac{1}{2}$ , $\frac{1}{2}$ , $\frac{1}{2}$
H %	
N '	$\frac{M}{M} = 2,626 + 0.622 \text{ F}$ $\frac{M}{M} = 493,100 + 0.217 \text{ F}$
	7E -01100 + 0 210 -
B	20.0 Hz () (4.5.
E	$\frac{M}{M} = 2,092 + 0.339 \text{ F}$
L	$\overline{M} = 0.111 \text{ p} + 0.339 \text{ F}$
·=	$M = 111 \Gamma - 5$
M	$\frac{\widetilde{M}}{\widetilde{M}} = \frac{8 + 0.234  \mathrm{F}}{1.234  \mathrm{F}}$
N %	$\frac{M}{2} = 502 \cdot 10^{-2.34}  \text{F}$
D or	$\frac{M}{M} = \frac{592 + 0.834  \text{F}}{466 + 0.834  \text{F}}$
В %	3E ±00 ± 0 241 =
E %	$\frac{M}{M} = \frac{27.5 + 0.382 \text{ F}}{3.5 + 0.382 \text{ F}}$
L %	$\frac{M}{2} = 35 + 0.002 F$
M &	$\frac{M}{M} = \frac{3.5 + 0.289 \text{F}}{0.131 \text{F}}$
M %	$\overline{M} = 0.01  \mathrm{F} - 0.9$
$\overline{\mathbf{M}}$	$\frac{M}{M} = \frac{19.5 + 0.540 \text{F}}{1.540 \text{F}}$
estimated mol-	$\frac{M}{40404}$
$\overline{M}$ = estimated male value $F$ = female value.	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
R = total red call	
LOTAL red - II	

R = total red cell count; W = total white cell count; P = platelet count; H =hemoglobin content; N = absolute neutrophil count; B = absolute basophil count; E = absolute eosinophil count; L = absolute lymphocyte count; M = absolute monocyte count; N % = relative neutrophil count; B % = relative basophil count; E % = relative eosinophil count; L % = relative lymphocyte count; M %

seem, therefore, that unless an unknown factor or factors were operating, the higher red cell and hemoglobin values in the male group were due to constitutional differences between the sexes. With regard to man there is convincing evidence that these two blood constituents are higher in the male than in the female, but so far as we know it has not yet been definitely shown that these differences are not due to environmental factors. It will be recalled that although all animals included in the experiment were virgin stock, the mean age of 26.0





Text-Fig. 1. Coefficients of correlation between the blood cell values of male and female rabbits. The upper figure gives the simple zero order coefficients and the lower figure, partial coefficients with age eliminated. The horizontal lines show the levels of significance (P = 0.01) and probable significance (P = 0.05).

R = total red cell count; W = total white cell count; P = platelet count; H = hemoglobin content; N = absolute neutrophil count; B = absolute basophil count; E = absolute eosinophil count; L = absolute lymphocyte count; M = absolute monocyte count; N % = relative neutrophil count; B % = relative basophil count; E % = relative eosinophil count; L % = relative lymphocyte count; M % = relative monocyte count.

weeks represents a sexually mature animal. What effect various physiological states such as pregnancy, parturition, and lactation might have on the blood cytology of the rabbit are subjects which we plan to investigate.

It should be pointed out that the observations herein reported were made on animals of various breeds. Since wide differences were noted between the mean blood cell levels of different breeds (2), it would be expected that the variance in an array of numerical values would be smaller when they represented animals of the same breed than when they represented different breeds. Thus the standard error of the mean values in Table II is probably larger than would be expected were the observations based on animals of identical breed. The method of analysis employed herein, that is, obtaining the mean difference between the pairs of values and the standard error of the mean difference, reduces the variation due to breed and tends to bring out the significance of smaller differences.

With the exceptions noted, the blood cell levels of bucks and sister does were similar. tion as to whether values for litter mate bucks and does ran parallel This method of analysis, however, gave no suggesto each other. Although the mean difference between two groups might be statistically insignificant, individual values for a pair might be totally dissimilar. Correlation coefficients and regression equations (Tables III and IV, Text-fig. 1) were, therefore, calculated in order to determine to what extent a knowledge of the blood cytology of one sex was relevant information concerning the blood cytology of a litter mate of the opposite sex. The significant correlations indicate that with respect to the red cell count, white cell count, hemoglobin in per cent, lymphocytes in absolute and relative numbers, and neutrophils, basophils, and monocytes in per cent, when high values are obtained for a buck, the expectation is that high values will also be found in its litter mate sister. Similarly, low values for the buck would be paralleled by low values for its sister. The extent to which age influences these findings is negligible since the elimination of age did not appreciably affect these relationships. It is of interest to note that all of the fourteen correlation coefficients are positive in sign, and that their summation by the method of transformed correlations yields a significant positive result  $(r_{xy} = +0.3908, P = 0.01-)$ .

The question arises whether these correlations measure the effect of environmental or hereditary factors, or a combination of both. experiments were not planned to elucidate this problem, and it shall. therefore, not be discussed in detail here. Attention, however, should be directed to the fact that a series of studies in this laboratory on the blood cytology of the male rabbit have been directed to this end (2). Employing the method of analysis of variance, significant differences between breeds were found for the following cell constituents: red blood cell, total white cell, and platelet counts, hemoglobin in per cent, basophils, eosinophils, and lymphocytes in both absolute and relative numbers, and neutrophils in per cent. It was concluded that these differences in the blood formula of normal rabbits were largely inherited. By the method of linear correlations employed in the present analysis, the following seven of these eleven blood factors gave significant correlations between the sexes: red and white cell counts, hemoglobin in per cent, lymphocytes in both absolute and relative numbers. and neutrophils and basophils in per cent. By the analysis of variance utilized in the former study, three blood factors, namely monocytes in absolute and relative numbers and neutrophils in absolute numbers, failed to show significant differences between breeds. In the present investigation two of these three factors, neutrophils and monocytes in absolute numbers, were not significantly correlated. Moreover, the most striking variations between breeds lay in the values for the red blood cell count, hemoglobin, lymphocytes, and basophils. present study, three of these four factors, red blood cell count, hemoglobin, and lymphocytes, gave the highest correlations obtained. The failure to obtain complete correspondence between the two methods of analysis, by variance employed in the previous study, and by linear correlations employed in the present investigation, was probably due to differences in breed and especially to the smaller statistical sample represented by the male and female groups. evidence, however, is sufficiently striking to conclude that the correlations obtained were largely measures of hereditary rather than environmental factors, and that the female rabbit as well as the male may be utilized in studying the mechanism of such inheritance.

It is of interest to note that the blood constituents which gave the highest correlations, namely red and total white blood cell counts,

hemoglobin, and lymphocytes, were found to be among the most reliable indices of the natural resistance of normal male animals to inoculation with a transplantable tumor (4) and to Tr. pallidum infection (5). It would appear, therefore, that the same constituents should furnish dependable information concerning the natural resistance of the female rabbit to these two diseases.

- 1. The blood cytology of 41 pairs of virgin rabbits was studied, each pair consisting of a buck and a litter mate sister. A comparison of the values for each blood factor of the two groups was made by obtaining the mean difference and the standard error of the mean difference. In addition, correlation coefficients, both zero order and first order eliminating age, and regression equations were calculated for each of the blood cell elements of the bucks and does.
- 2. It was found that the male values for both the red cell count and the hemoglobin content were higher than the corresponding female values, and these differences were of statistical significance. No differences were observed between the two groups with respect to other cells. Significant positive correlations were obtained between the two sexes for the following blood constituents: Red blood cell count, white blood cell count, hemoglobin content, lymphocytes in both absolute and relative numbers, and neutrophils, basophils, and monocytes in relative numbers.
- 3. Since all known variables had been held constant, it was concluded that the higher red cell count and hemoglobin value for the male group were the results of constitutional differences between the sexes. Moreover, the significant correlations between the blood cells of male and female litter mates were ascribed to hereditary influences, and thus the female as well as the male rabbit is available for studies on the mechanism involved in the inheritance of the blood formula. In addition, since high correlations were obtained for cells which have been found to be the most reliable indices of the natural immunity of the male host to subsequent inoculation with tumor or syphilis, it was concluded that these cells should give dependable information concerning the natural resistance of the female host to

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### A VIRUS-INDUCED MAMMALIAN GROWTH WITH THE CHARACTERS OF A TUMOR (THE SHOPE RABBIT PAPILLOMA)\*

I. THE GROWTH ON IMPLANTATION WITHIN FAVORABLE HOSTS

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(From the Laboratories of The Rockefeller Institute for Medical Research)
PLATES 36 TO 40

(Received for publication, August 9, 1934)

Efforts to demonstrate the presence of causative agents in the recognized mammalian tumors have to the present been unsuccessful. The work to be reported here was undertaken, not as a renewed attempt in this direction, but to determine whether a growth known to be caused by a virus and of endemic occurrence, namely the Shope rabbit papilloma (1), possesses the immediate characters and the potentialities of a tumor.

The growth, as found on the skin of wild rabbits, has the appearance of a papilloma and is devoid of inclusion bodies such as indicate the presence of a virus (2). During its early extension it invades downward until it meets obstruction; and, though ceasing to enlarge after a time, it seldom retrogresses. It is readily produced in domestic rabbits by inoculation of the virus; yet from the growths in such animals this virus cannot ordinarily be recovered in active form. As Shope has pointed out, an extraneous cause can no more be demonstrated under such conditions than in the mammalian tumors thus far tested or, for that matter, in chicken tumors of filterable origin when they are growing slowly. These traits of the growth, in special, have commended it as material for investigation.

Our experimental comparison of the papilloma with the recognized neoplasms will be divided into three parts for reasons of expediency, with frequent references in one part to the material of another, and consecutive numbering of the figures. The appearance and behavior

<sup>\*</sup> Reported in abstract before the Association of American Physicians, May 1, 1934.

of implantation growths in favorable hosts are the main themes of the present paper.

The papilloma is frequently encountered on the skin of "cottontail" rabbits in Iowa and Kansas, as one or more verrucous masses or cutaneous horns. Our supply of virus-containing material was generously provided by Dr. Shope. It consisted of pieces of papillomatous tissue from two individuals, and had been kept in a single bottle of 50 per cent glycerin at refrigerator temperature for nearly a year. We have drawn upon it for virus at intervals during 7 months, always with positive results. To prepare a virus-containing extract the fragmented tissue is put through three changes of Tyrode solution, with a stay of some minutes in each; ground with sand; and made up with Tyrode to a 5 or 10 per cent extract by weight. Sometimes this has been allowed to sediment briefly before inoculation, but in most instances it was centrifuged and a portion of the slightly cloudy fluid, virus fluid is it will be termed, was withdrawn through a long needle from the midst of the pernatant column. For routine production of the papilloma it was rubbed into the shaved skin of the abdomen immediately after light scarification of a broad expanse with sterilized sandpaper; but when the effect on the growth of various factors was to be tested several widely separate inoculations were made along the sides of the animal, either by intradermal injection of 0.1 or 0.2 cc. of virus fluid at each situation, or by tattooing through a drop of it placed upon the skin. An electric tattooing machine was employed, having a group of needles about 2 mm. across that could be readily sterilized in boiling water. The sites of punctate inoculation were marked by tattooing India ink into the skin near them, and charts were made of their precise relation to the ink spots.

For most of the work, adult domestic rabbits weighing about 2 kilos, of graybrown (agouti) breed, were utilized. The papilloma regularly develops in these when the virus is rubbed upon skin that has been scarified sufficiently to bring out a blood-tinged serum; and the growth tends to appear at about the same time in all receiving the same material. The incubation period from group to group ranged between 7 and 30 days, after broadcast inunction of the virus on the abdomen. To date we have thus inoculated 64 domestic rabbits, and the growth has appeared in every one. After intradermal injection, 1 to 2 months or more may elapse before the first papule is noticeable, and the results of some injections, perhaps a third in all, have been negative. Tattooing virus into the skin was found to yield the growth sooner and more consistently, doubtless because of the multiple inoculations effected with the needles.

The cottontail rabbits employed (genus Sylvilagus) came from Kansas. The growth was already present in two amongst 48 of them received, and these two proved resistant on inoculation with the virus, as did two others of the 25 thus far tested. The skin of cottontails is so thin that intradermal injections with viruscontaining fluid cannot readily be effected. Consequently tattooing has been done when multiple tests upon a single animal were desired.

Material fixed in acid-Zenker and colored with methylene blue and eosin, or with Giemsa's stain, has been used in the histological studies.

#### General Character of the Growths on the Skin

The histology of the growths on the skin has been described by Hurst (2). Little need be said of it at this point.

The virus causes a lively proliferation of the epithelial cells by mitosis in the basal layer and the rele Malpighii, with abnormal maturation and keratinization. The growing epithelium at first extends sideways under the neighboring epidermis and downwards through the connective tissue until it meets the fibrous corium, thereafter protruding in papillae that often become greatly elongated. The proliferating cells are far larger than normal, appear darker with methylene blue, have much larger nuclei, and the granules characterizing the granular layer tend to be especially abundant, large, and intensely staining. The cells flatten much less than normally during differentiation, and they fail to desquamate after keratinizing. Furthermore the papillae dry at their summits after a time because their blood supply is interfered with by lateral pressure. In consequence of these processes the growth soon becomes capped with a thick, firm layer of desiccated material, which may build up to a height of several centimeters, forming a cutaneous horn. Sometimes its living portion is pigmented, not infrequently almost coal black in the gross. The color is due to brown or black, fine or coarse granules of pigment situated in the epithelial cells and also in chromatophores scattered amongst them and in the immediately underlying connective tissue. Apart from the presence of these granules, the pigmented growths look like the others. The conditions determining pigmentation will be the subject of a future paper. The normal epithelium of the skin of cottontails and of domestic gray-brown rabbits such as we used contains brown pigment granules.

The morphology of the papilloma produced by rubbing the virus into scarified skin differs somewhat in wild and domestic rabbits. In the former it appears as pink or gray papules which darken as they enlarge, and the growth soon consists of a broad, elastic mass of nearly black, big and little, more or less fused cones with plump bases (like onion sets) resting on a thin layer of connective tissue. The mass is on the average ½ to 1 cm. high, showing far less tendency to build up than in domestic rabbits. In the latter the growth is relatively exuberant and fleshy, and may be pink or sooty, low or projecting, dry or succulent. When virus has attacked the epithelium at many neighboring points in a scarified area, with result in multiple papillomas, these soon coalesce into a mass; whereas if the initial change has been confined to one spot, as on intradermal inoculation, a single, sharply circumscribed papilloma develops. According as this broadens rapidly or slowly, or

VIRUS-INDUCED MAMMALIAN GROWTH. · I undergoes secondary constriction at the base, it becomes verrucous or cone-shaped, perhaps acuminate, or even tassel-like with a peduncle. In masses formed by secondary fusion of many small, contiguous growths, portions of normal skin with their appendages are frequently carried far above the surrounding surface. In consequence some growths become covered secondarily with thin hair. Nipples with unaffected epidermis may occasionally persist for weeks at the summit of high growths, before their blood supply is interfered with and they undergo the general drying.

Though capable of affecting the cells of an alien genus of rabbit the virus produced no lesions on the skin of the rats, mice, or guinea pigs that we inoculated; and inoculations into four cats, one pig, and one goat have also yielded negative results. In susceptible animals the virus is notably specific for the epidermis.

Virus fluid was rubbed into large areas on the sides of several domestic rabbits, and small strips of the inoculated skin were removed daily, under ether, throughout the period until the growth appeared. Microscopically it could be seen that the scarification had broken through the epithelial layer, as a rule almost midway between the orifices for the hairs, and here after the break had healed the characteristic epithelial proliferation first appeared. Often, especially in experiments involving intradermal inoculation, the virus must have been brought into contact with the Cells of traumatized hair follicles and sebaceous glands, yet we have never encountered a growth with morphology suggesting an origin from these structures. The epithelium of the necks of the hair follicles sometimes showed proliferation and the cellular changes characteristic of the virus, but the cells of the hair bulb were always unaffected. The typically differentiated cells of the sebaceous glands may come to be surrounded and underlain by a layer of the changed epithelium, but they are readily distinguished therefrom. Whether this layer represents an intrusion, or follows upon virus infection of the basal layer of the sebaceous gland, with result that its cells proliferate without differentiation, remains to be determined.

The virus often fails to give rise to the papilloma when tattooed into the ears of wild rabbits or does so only very slowly, though promptly causing the growth when introduced elsewhere in the skin of the same individuals. This may be due to the toughness and tenseness of the tissue, which will not admit much extraneous matter on the tattooing needles; for takes are readily got on the softer skin of the ears of domestic rabbits. Inoculations into the prepuce of a wild rabbit,—which was susceptible as shown by the results of a skin inoculation made at the same time, and into the prepuce, tongue, mucous membrane of the cheek, and hard palate of two domestic ones resulted negatively. So, too, did direct injection of virus fluid into the liver, spleen, stomach, appendix, cecum, rectum, and kidney of an etherized wild rabbit later proven susceptible, and into the liver, kidneys, stomach, and the a submaxillary gland of two domestic animals. In each case one ureter and the

duct from one submaxillary gland had been tied some weeks previously with a view to rendering the gland epithelium more susceptible. The gastric mucosa and that of the intestines were repeatedly needled while injecting the virus, to bring it into direct contact with them. In two wild rabbits the lungs and trachea were inoculated by thrusting the injecting needle into them here and there through the cleansed skin of the etherized animal. When they were sacrificed, after 66 and 67 days, no lesions were found anywhere save on the skin where the needle had been thrust through. Here in one instance the papilloma had developed. Shope found the virus ineffective when injected subcutaneously or into the peritoneal cavity; while when introduced into the blood stream it produced lesions only in the skin, at points of injury.

The agent responsible for Chicken Tumor I causes tumors most readily in young birds, the embryonic mesoderm being highly susceptible to it (3). Because of this fact we have explored the possibility that the papilloma virus might act upon embryonic epithelia of various sorts.

Three experiments were done, with material from rabbit embryos 10 to 26 days old from domestic does. The lungs, kidneys, livers, intestines and stomachs, brains, placentas, and skin were removed separately, finely hashed, and placed in suspension in Tyrode solution. A hash of the skinned embryo was also used in one case. Half of each suspension was mixed with an equal amount of freshly prepared 5 per cent papilloma virus in Tyrode solution, while the other half, appropriately diluted with Tyrode, served as control. The mixtures were allowed to stand 30 minutes at room temperature in two experiments, 2 hours in another, with frequent agitation, and were then implanted subcutaneously along the sides of twelve domestic rabbits of the same breed, 0.2 cc. at each site, the virus-containing suspension on one side, the control suspension on the other. When necessary to obtain sufficient material, organs of the same kind from several embryos were pooled.

The bits of placenta, kidney, liver, and brain did not long survive in the new hosts, but those of skin, stomach and intestines, lungs, and whole embryo proliferated, forming little nodules, and these were found to contain much living epithelial tissue of the characteristic sorts when the animals were killed after 1 to 3 weeks. The virus had had no discernible effects on this tissue, which resembled microscopically that in the control nodules on the other side of the host. In one instance two spherical lumps about 0.8 cm. in diameter developed just beneath the surface along the track of the needle used to inject embryo skin mixed with virus, but several centimeters away from the little nodule developing from the latter,—which itself showed no virus changes. They consisted of proliferating papillomatous tissue such as results from subcutaneous implantation of the papilloma, as described further on. They probably arose from epidermal cells of the host, pushed beneath the surface by the needle and at the same time inoculated with virus.

VIRUS-INDUCED MAMMALIAN GROWTH. I The Growths Resulting from Transplantation There are histological indications that the papilloma can invade the surrounding structures on occasion. It not only grows downwards when first appearing but later may thrust short processes into the underlying connective tissue. This gradually thickens, often with small accumulations of polymorphonuclear leukocytes here and there in it, and after some weeks a dense layer of scar tissue has developed. The epithelium of the growth continues to proliferate but now the latter no longer enlarges. In order to determine how it will behave if placed under different conditions, as also whether the inflammatory reaction present beneath long established growths is characteristic or consequent merely on trauma and infection, we have implanted bits of papillomatous tissue in various organs of the host.

Melhod.—The skin of the abdomen of eight domestic rabbits and four wild ones Was scarified over a large area and virus-containing fluid was rubbed in, with result in a confluent, papillomatous change. When the broad "pancake" of new-formed papillae was 1 to 2 mm. high, but still everywhere living and soft, it was vigorously Scrubbed with soap and water, rinsed, and dried with sterile sponges. The surface layer was removed with a razor and discarded, and then a shaving like a Thiersch graft was taken off into Tyrode, cut into fragments small enough to pass through an 18 gauge needle, and injected in suspension into the extensor muscles of the fore or hind legs of the rabbit furnishing the material and into the liver, spleen, kidney, stomach, and the subcutaneous tissue of axilla or groin. A laparotomy was done under ether to reach the viscera.

All of the animals receiving implants of the papillomatous material have developed growths, often enormous ones (Figs. 1, 36, 37, 38). Some of them were killed for examination; the others have become cachectic and died, usually from a terminal infection. The growths progressed more rapidly in wild rabbits, one succumbing to them in 39 days and another in 81 days. The domestic rabbits died of them after 94 to 115 days. For some time before death the "pancakes" of papillomatous tissue providing the material for implantation had been stationary in size, and indeed one was retrogressing. General Findings.—The implantation growths had the same general Structure in all the organs (Figs. 2, 3, et seq.). They were rounded or irregular, according as they had developed from a single focus of growth or from a number, well demarcated, creamy, gray or even

black, or patched with gray or black (Figs. 2 and 3), resistant to the knife, close-textured, and necrotic everywhere except in their outermost layer and along some inward extensions from it. Many had an evident connective tissue capsule while others had none, at least in certain regions. The papillomatous structure was faithfully recorded in the necrotic tissue, which was tough and rather dry; but the papillae did not project outwards like those on the skin. Instead they extended toward the center of the growth, with their base at its periphery (Figs. 8, 12). This arrangement had resulted, not from a buckling inwards of the proliferating epithelial layer,—for the dead interior of the mass was notably dense,—but from a direct penetration of the surrounding tissue by blunt epithelial processes which advanced in a more or less orderly fashion so close together that only a thin layer of tissue lay between adjacent ones. The proliferating basal epithelium formed the outermost layer of the growth, the dead, keratinized cells accumulating in its interior. The pattern was still papillomatous but now with papillae turned in. The septae were derived from the growth's surroundings, both by inclusion between the epithelial processes and by secondary changes in the included tissue, such that it became loose-textured, and its vessels wide, numerous, and thinwalled. The proliferating activity of the epithelial cells greatly exceeded in general their ability to thrust their columns into the surrounding structures, and in consequence most of the mass was necrotic. The papillae within it, nourished only by the vessels of their narrow cores, died en masse as their blood supply was cut off by the pressure of their own proliferation, though occasionally living ones persisted almost to the center.

Growths in the Subcutaneous Tissue.—The numerous implantations into the loose tissue of axilla and groin have yielded nodules that sometimes reached a diameter of 5 or 6 cm. and interfered with locomotion.

The growths were roughly spherical (Fig. 1) or like several coalesced spheres, elastic and well encapsulated, with occasional blunt protuberances. On section they showed a rind of translucent, grayish pink, living tissue about a dense, creamy to black, papilliform or concentrically striated, necrotic mass (Fig. 2). Very occasionally, as in other interior situations, irregular clefts existed between the papillae, containing a colorless, watery fluid (Fig. 10). There was a more or less well defined capsule of young connective tissue cells, with sometimes a few round cells but no

polymorphonuclear leukocytes; and the capsule and the surrounding connective tissue were far less compacted than if the growth had enlarged by expansion only. The lymph glands frequently became attached to the capsule but in general were not invaded. Sometimes as the growths became large the skin over them broke down and necrotic papillomatous tissue was exposed.

Muscle Growths.—The voluntary muscles proved a more favorable situation, the growths developing faster, with much invasion and relatively little encapsulation (Figs. 2 and 18 to 21).

The extensor muscles of the upper legs were utilized for implantation, preferably those of the forelegs since here small nodules could readily be palpated. The bundle of muscles was held between the thumb and forefingers, with the shaved skin drawn tense, and 0.5 to 1.0 cc. of a thin suspension of papilloma fragments was injected into it at a single point. After 2 to 3 weeks, one or more little nodules could be felt at the immediate site of injection, and these rapidly enlarged. The growing mass frequently replaced almost wholly the extensors in which it lay, occupying the space from elbow to shoulder; but the uninvolved muscles persisted as an overlying, thin layer. In domestic rabbits dying of the papilloma 3 to 4 months after implantation of it into the viscera and forelegs, enormous masses had developed in the latter situation (Fig. 37). They sometimes extended almost Completely around the humerus (Fig. 38), causing a partial paralysis with toe-drop

and a further limitation of motion due to encroachment on the axillary space. The muscle growths had usually (Fig. 37) though not always (Fig. 1) a capsule Visible in the gross. When rapidly enlarging, in wild rabbits especially, they were irregularly football-shaped with their long axis in the direction of the muscle fibres, and on cross-section they appeared to consist of many small, coalescing nodules (Fig. 2). These were the consequence of a thrusting forth everywhere at the periphery of the mass of proliferating epithelial processes which became nodular foci of growth while themselves thrusting forth new processes. Gross section showed numerous, discrete patches of dry necrosis scattered amidst grayish pink or sooty, translucent tissue that under the microscope proved to be actively proliferating epithelium, more or less encapsulated by new-formed connective tissue (Fig. 18), but invading the latter and not infrequently extending beyond its limits. The enlargement of the growth was in considerable part the result of invasion. Sometimes there was a direct penetration of the epithelium amidst the muscle fibres, with replacement of them (Figs. 19, 20, and 21). The histological appearance of the stat ance was very different from that due to proliferation of the sarcolemma sheath with giant cell formation, so frequently seen in injured muscle.

Growths that progressed slowly were spherical or egg-shaped (Fig. 37), or as if composed of several partially fused spheres, smooth-surfaced, well encapsulated, notably firm, and necrotic save for a thin rind of the characteristic epithelium (Fig. 38). The necrotic material, which was sometimes putty-like, shelled out

readily, leaving a smooth or velvety, living layer with small papilliform projections here and there. Such growths resembled those resulting from implantation in the relatively unfavorable subcutaneous situation.

Liver Growths.—Nodules regularly developed at the implantation sites in the liver, and sometimes nearby,—doubtless from small fragments accidentally introduced into blood vessels with the injection fluid, as will in due course appear.

The growths were spherical or irregular, often coalescing, and creamy to sooty in hue (Figs. 1 and 3). They usually protruded above the liver surface, and often reached a diameter of several centimeters (Fig. 36). On section they stood out from the parenchyma, and were firm, close-textured, necrotic save at the periphery. and relatively bloodless. The sooty regions were sharply marked from the adiacent creamy tissue by their hue (Fig. 3) but not in other ways. Microscopically the characteristic, introverted papilliform arrangement was found, with a rind of actively proliferating epithelium and an occasional living papilla far in the interior. The sooty regions showed pigmented epithelial cells and chromatophores. As in the case of growths in the subcutaneous tissue and muscle, round cells and polymorphonuclear leukocytes were notable by their absence; but there was ordinarily an irregular encapsulation with new-formed connective tissue, which was thin in some places and in others wholly lacking. Where it lacked there was no cellular reaction and the interlobular blood sinuses alone separated the liver cords from the proliferating epithelium (Figs. 8 and 11). The arrangement of the latter was orderly as a rule, growth being largely expansive, though taking place also from processes pushed here and there into the parenchyma. Not infrequently, especially in wild rabbits, narrow, disorderly tongues of epithelium extended out and directly replaced the liver cords (Fig. 11).

Splenic Growths.—These were like the ones in the liver. They grew both by expansion and by direct invasion and replacement of the splenic pulp.

In Fig. 12 two of the invading tongues of epithelium have differentiated into pearls. Mitoses were often exceedingly numerous (Fig. 9), as in the liver. There was ordinarily little or no encapsulation (Fig. 12) or other reactive change, and while sometimes the surrounding parenchyma had been compacted by the growing mass in most instances it had not. Often the short, thick spleen of wild rabbits was almost wholly replaced by the growth, only its ends remaining as tails attached to a spherical nodule 2 cm. or more in diameter (Fig. 1). In one case a thin skim of ruddy parenchyma partly covering a spherical growth 3 cm. in diameter was all that remained of the spleen. In the elongated, firm organ of domestic rabbits replacement was far less complete.

Growths in the Stomach.—The implantations in the stomach were often faulty, with escape of some at least of the fragments into the lumen of the organ or into the peritoneal cavity; and not infrequently the papilloma failed to develop at the injection site.

In positive instances an encapsulated, spherical nodule was found at autopsy. Usually it was not more than 0.3 to 0.7 cm. in diameter, even when the liver growths were several times this size. Microscopically it had the typical arrangement. Occasional epithelial processes extended into the surrounding capsule but there was no direct replacement of the gastric tissue. Sometimes the growth protruded on the peritoneal surface (Fig. 36) but more often into the lumen of the stomach. The overlying mucosa was usually unchanged, but sometimes had undergone a local thinning or showed hemorrhages or scarring.

Renal Growths.—In the relatively firm kidney cortex the papilloma grew slowly, as one or several rounded nodules (Fig. 4) surrounded by new-formed connective tissue, perhaps with some round cells. However it not infrequently put forth processes which penetrated irregularly between the tubules. A differentiation to pearls can be seen in Fig. 7.

Where the growth reached the kidney surface, and in consequence was relieved from pressure, it rapidly enlarged with result often in a projecting sphere. In one cottontail a narrow, pointed, brown horn was found projecting into the kidney Pelvis. Conical and smooth, it was about 6 mm. long and 2 mm. across at its base on the medulla. Serial sections showed that it had arisen where a papillomatous nodule in the kidney substance opened out directly upon the pelvic surface. It Consisted of keratinized epithelium everywhere except at its proliferating base (Fig. 49).

# Enhancement of Malignancy by Bacterial Infection

The implantations into the leg muscles were carried out not only for their own sake but to provide material free from bacterial contamination for later transplantation to other individuals. perience of one of us with ulcerated, spontaneous chicken tumors has been that if portions are transplanted to other fowls death or local necrosis is the usual consequence, whereas implantation into the muscle of the host is well tolerated, and bits of the growths to which The exit gives rise can be introduced into other individuals without danger. Presumably the host has developed an immunity to the organisms contaminating the tumor, which suffices to destroy most of those

introduced with pieces of it. However this may be the fact is certain that the intramuscular injection of pieces of papilloma from the skin seldom resulted in any evident infection, although innumerable bacteria must have been introduced with the material. The resulting growths enlarged by expansion, by the thrusting forth of orderly processes, and to a minor degree by irregular invasion. Occasionally, however, acute inflammatory changes took place about the implanted bits, with accumulation of pus cells and necrosis of the papillomatous tissue. Bacteria were present as stains showed. In other cases a chronic, more or less widespread, interstitial cellulitis developed, with The papilloma growing amidst tissue affected in the latter way showed an active malignancy, sending out long, thin, disorderly tongues of proliferating epithelial cells which thrust between and around the individual muscle fibres, destroying them by pressure or contact (Figs. 19 and 20), and in some cases penetrating within the sarcolemma and directly replacing their substance (Fig. 21).

The rabbit of the instance figured had received implants into the liver, kidney, and spleen at the same time with those into the muscles. It died on the 23rd day after implantation, of subacute peritonitis and pleuritis. The growths in the viscera were actively invasive. Such slight cellular reaction as occurred about them did not suggest an associated infection. This has been a general finding with visceral implants of material which, in the muscle, gave rise to cellulitis.

#### Recurrence and Operative Dissemination of the Growth

On numerous occasions intramuscular growths have been removed from the leg for transplantation or study; and the attempt has been made to take them out completely, though with the least possible excision of the normal structures. They have nearly always recurred, not only in the muscle but sometimes in the subcutaneous tissue that was cut through; and their subsequent growth has generally been more rapid and extensive than that of the control mass in the other leg.

The laparotomy for implantation in the abdominal organs was usually made well to one side of the abdominal "pancake" of papillomatous tissue furnishing the material, but in some instances was tangential to it. Under the latter circumstances skin growths previously stationary extended for some millimeters along the newly healed incision, but not elsewhere. No independent papillomas, such

as might have resulted from secondary infection with the virus, appeared in the skin incised during the process of implantation, save in a single instance of one new growth; and the derivation of this was rendered uncertain by the development of papillomatous nodules in the underlying subcutaneous tissue and healed muscle, obviously as result of an accidental scattering of tissue bits at the time of operation. Operative dissemination often resulted in nodules on the peritoneum. During the injections into the viscera some of the papilloma fragments nearly always escaped into the abdominal cavity; and the nodules eventually found attached to the peritoneum were composed of epithelium like that introduced. Mention has already been made of the fact that intraperitoneal inoculation of the virus as such fails to cause lesions.

The peritoneal implants (Fig. 36) were usually few, never more than fifteen, and their size and condition indicated that most had been developing for a considerable period, doubtless from the time of laparotomy. Contrary to expectation, none had the frankly papilliform shape of the skin growths. They were always rounded, often with but a small base, and they had the introverted arrangement. Peritoneum, and as a rule connective tissue, covered them completely even when, as sometimes happened, they were attached by but a slender pedicle or cord. The proliferating rind of living epithelium was in many instances a smoothly curving layer, or but slightly papillomatous; but here and there it thrust processes into the connective tissue, and these were often irregular and invasive (Fig. 15).

The growths developing in the muscle along the healed laparotomy wound had the same morphology as those due to direct implantation in the leg muscles. frequently pushed into the peritoneal cavity secondarily, and here the release from pressure caused them to mushroom out (Figs. 1 and 5); but always the broad, mushroom cap was found to be covered with peritoneum. The living layer of epithelium in the most projecting portion of the cap was frequently very thin; and occasionally both it and the peritoneum gave way before the interior pressure, and there resulted a secondary bulging sphere, with sometimes a third, smaller one upon that, the result being a conical mass made up of spheres of diminishing size, one on top of another, like a Tibetan monument. This was sometimes the case also when kidney or stomach growths had emerged on the peritoneal surface. Occasionally the connection of a secondary protrusion with the parent nodule was very narrow, like a bud (Fig. 32); but it is doubtful whether such buds ever came away and gave rise to growths.

Many mouse tumors fail to "take" when bits of them are injected into the abdominal cavity, but they will do so if introduced with an

irritant such as diatomaceous earth which aggregates here and there on the peritoneum and induces locally a connective tissue reaction favorable to the tumor cells,—as has been proved in a study of the reasons for the localization of metastases at points of injury (4). The findings just described show that the papilloma can implant itself on the peritoneal lining without such aid; but nevertheless bits of it, from the abdominal "pancakes" of domestic rabbits, were placed in suspension with diatomaceous earth and injected into the peritoneal cavity of the hosts. In some cases, though not in all, implantation growths resulted; and these had the characteristic morphology, and most of them had arisen where the diatomaceous earth elicited a reactive proliferation of the connective tissue.

#### Implantation by Way of the Blood Stream

Can bits of the papilloma give rise to the growth when distributed on the blood stream? Tests of this possibility were made.

Four domestic rabbits were used. The material for injection into two of them was obtained by slicing off and finely mincing in Tyrode parts of their own early skin papillomas, filtering the suspension afterwards through gauze to remove large fragments. In the case of the other two, muscle growths resulting from implantation were excised under ether and the rind of living tissue was treated in the same way. Muscle growths were used because it seemed possible that the bacteria present on skin material utilized directly might cause death of the inoculated fragments. From 2 to 4 cc. of thin suspension was injected into an ear vein of each rabbit; and from 2½ to 10 cc. into the central artery, in the direction of the heart,—after the vessel had been distended by heat and the large marginal veins had been temporarily closed with rubber-covered clamps to prevent a short-circuiting of the material into the venous blood. The arterial injections were done rapidly under high pressure, with a view to obtaining distribution by way of the aorta.

One of the rabbits was killed 13 days after injection. Its lungs contained numerous scattered, creamy, spherical nodules, from \( \frac{1}{2} \) to 2 mm. in diameter. None was found in the other organs. The pulmonary nodules had the characteristic morphology of the growth, some consisting of a rind of more or less orderly epithelium enclosing concentric layers of necrotic squamous tissue, while others were made up of living tissue almost throughout, and had the papillomatous form (Fig. 13). At the periphery of the latter the epithelium had pushed into the individual alveoli in disorderly aggregates, filling and destroying them (Fig. 14). There was some compression of the surrounding lung tissue, and the neighboring small bronchioles were more or less flattened; but not the least encapsulation of the growths

had occurred, nor any inflammatory reaction. There were many mitoses, and the characteristically pathological epithelial differentiation and keratinization.

The findings were negative in another rabbit, killed after 14 days; but in one of two that were allowed to live 3 months scattered pulmonary nodules from 3 to 7 mm. in diameter were found, of characteristic morphology. The other organs were The rabbit had received bits of an intramuscular growth into the circulation.

# Interpretation of the Results of Implantation

The morphology of the nodules developing in the interior of the host as result of the injection of tissue fragments; their frequent situation in tissues from which they could not possibly have been derived by direct virus infection, as e.g. muscle and spleen; the evidence for accidental implantation of the papilloma cells on the peritoneal surface, and in the muscle and subcutaneous tissue of wounds; and the specificity of the virus for epidermal cells, together provide conclusive evidence that survival and proliferation of the engrafted tissue was responsible for the growths. Final proof that the lung nodules which appear after injection of papillomatous tissue into the blood stream result from its survival and proliferation must wait upon a study of serial sections of early stages in their development. But the demonstrable ability of the papilloma cells to multiply when implanted elsewhere in the host, the rapid development of the nodules, and the failure of the virus to cause lung growths when injected as such into the blood stream or needled directly into the pulmonary tissue leave small doubt that this was the case.1

The liver, spleen, and voluntary muscles seem most favorable to grafts of the papilloma. When growing in the connective tissue it is well encapsulated, and pressure conditions in the kidney appear to limit it to a considerable degree. In the stomach wall it does poorly for reasons not yet plain. The nodules developing in the lungs have been few compared with the number of tissue fragments injected into

Serial sections now at hand have yielded direct proof that papilloma fragments lodging as emboli within branches of the pulmonary artery survive, proliferate, penetrate the vessel wall, and rapidly invade the lung parenchyma. Nodules 1 mm. in diameter may develop within 5 days after the intravenous injection of tissue; and all are due to the growth of emboli. The lung epithelium itself undergoes no proliferation.

the circulation, and none have appeared in the other organs, though the material must have been distributed to them on the arterial blood. Even in the most favorable situation the growth's invasive power fails to equal its ability to proliferate.

Usually some new formation of connective tissue takes place about the papillomas developing from implants, its amount varying inversely with the rate of growth. When this is rapid, as in certain viscera, there may be no visible cellular reaction about the advancing epithelium (Figs. 11, 12, 13, 17). This holds true of not a few skin growths as well, at the period of their early, downward extension (Paper III). It is plain that the papilloma does not inevitably call forth reactive changes on the part of the surrounding tissues; but how far those ordinarily associated with it are due to bacterial infection is still uncertain. The scarring frequent beneath old skin papillomas is certainly due to this cause, and so too are the focal accumlautions of polymorphonuclear leukocytes. They are missing from about implantation nodules save when bacteria are present. Fewlymphocytes are to be seen save when retrogression is taking place (Paper II). All in all the facts suggest that the papilloma as such elicits some connective tissue proliferation over and above that incident to the formation of a stroma, its amount depending on both the local conditions and the time available,—the epithelium sometimes advancing so fast as to outstrip any formation of it. The behavior of the growth as conditioned by the reactive tissue round about will be considered in Paper II.

#### The Invasion of Vessels

The cores of the papillomatous projections from the skin contain many wide, thin-walled vessels, and often the proliferating epithelium is separated from the blood by only a layer of endothelium. Direct invasion of the vessels takes place frequently, and may occur even when the growth is progressing very slowly.

The lymphatics beneath the cindery, almost stationary papilloma of Figs. 28 and 30 were invaded at several points; but the cells that had entered were differentiating and dying. In Fig. 16, taken from a growth in the abdominal wall, the epithelium within the lymphatic is also in poor condition, though the growth from

which it had extended was proliferating actively. Fig. 15 comes from the periphery of an accidental peritoneal implant; and it shows epithelium that has entered a blood vessel, covered with platelet clot. Serial sections demonstrated the fact that the invading cells represented the tip of a downgrowth from the main nodule, part of which can be seen near by.

## The Possibility of Metastasis Formation

The frequency with which the papilloma invades the vessels, and the results of injecting it into the blood stream would lead one to expect metastasis formation in especially favorable hosts. been made to induce it (for which see Paper II); and in every animal coming to autopsy search has been made, microscopic as well as gross, where this seemed advisable, for secondary localizations in the lymph Efforts have nodes and lungs. A growth was found once in a lymph node (Fig. 17), but the possibility that it resulted from operative introduction of papillomatous tissue into a regional lymphatic cannot be excluded. In two instances growths were found in the lungs (Fig. 6), but also under equivocal conditions.

The domestic rabbit yielding the growth in the lymph node had been subjected to a variety of procedures. The papilloma virus was tattooed into four spots on each side and rubbed into a scarified area on the abdomen. 9 days later when the growth was first appearing in the latter situation part of it was shaved off and implanted by the usual methods in the muscles of the hind legs, the subcutaneous tissue of the left axilla, the liver, and the right kidney. Some of the papillomas appearing later where the skin had been tattooed were kept covered with a layer of collodion, and grew beneath the surface in consequence (Figs. 22 and 24); whereas the tissue under and about others was repeatedly infiltrated with olive oil containing Scharlach R in saturated solution, with result that they developed into large, fleshy, subepidermal masses (Figs. 22 and 23) which eventually fungated and became foul. One of each sort was submitted to biopsy. The "pancake" on the abdomen was redundant and fleshy at the time when the animal died, 94 days after the implantations into the viscera, which had all given rise to large growths. There was a spherical mass, 3.5 cm. in diameter and characteristically papillomatous where the graft had been placed in the left axilla; and near by but wholly separate, a lenticular growth 1.5 cm. across, which had appeared late and on repeated palpation during life had given the impression of developing in a lymph gland. Section showed this to be the case. The gland was entirely replaced by papillomatous tissue save at one end. The epithelial processes were highly irregular and very invasive (Fig. 17).

In the lungs of the animal were three papillomatous nodules, from 4 to 10 mm. in diameter, rounded, creamy, and projecting. They were all in the right upper lobe (Fig. 6). Gross and microscopic examination disclosed no others.

In this rabbit the papilloma grew especially well. Some large nodules were found at autopsy, scattered on the peritoneal surface, and there were several in the scar of the old laparotomy wound. The situation of the lung nodules in a single lobe, their large size, and the absence of any early, small ones suggest that they originated from cells accidentally introduced into the blood stream during the injection of the liver with suspended papilloma particles. The growth in the lymph gland may have been a metastasis, or may have arisen from cells introduced into the lymphatic stream when the axillary implantation was made or during biopsy of one of the stimulated, fleshy growths situated beneath the surface of the side.

In another domestic rabbit, dying 115 days after implantation into the liver and other organs, a single pulmonary nodule 7 mm. broad was found. The presence of large growths on the peritoneum and in the old wound indicated that this animal, like the one just discussed, had been favorable to accidental dissemination of the papilloma (Fig. 36).

Whatever the method whereby the growths developed in lymph gland and lungs, whether by an operative scattering or by true metastasis, there would seem to be no doubt that they arose from cells transported on the lymph and blood streams.<sup>2</sup>

#### The Recovery of Virus from Implantation Growths

No difficulty has been experienced in the recovery of virus from implantation growths in the muscle and viscera of wild rabbits. Both fresh and glycerinated tissue (50 per cent glycerin) have been employed, with grinding and extraction in Tyrode as usual, and inoculation into the scarified skin. Two experiments of the sort, with positive outcome, are described in Paper III, in connection with the work on transplantation. A third, similar experiment, but with material from a domestic rabbit, yielded negative results, as was to have been

<sup>2</sup> In the pulmonary alveoli of cottontail rabbits with pigmented tumors in the liver, aggregates have been found of cuboidal cells having much the same general appearance as those of the hepatic growths, and like them containing brown or black pigment. But though such aggregates are always very small, no signs of an origin from cell emboli have been noted; the cells rarely show mitoses; and they sometimes desquamate and become foamy elements like *Herzfehlerzellen*. Furthermore they can be found in the lungs of wild rabbits that are not carrying the papilloma.

expected, since the virus cannot ordinarily be recovered in active form from skin papillomas experimentally induced therewith in such animals.

Effects of the Growth on the Host No sufficient study has yet been made of what happens to the skin papillomas eventually or of their ultimate effects on the host. cells certainly can proliferate for long periods: they were still doing so after 13 months in the case of some of Shope's domestic rabbits. Growths of large size on the skin of such animals, resulting from inoculation of the virus into a broad area of scarification, are well tolerated but lead frequently to secondary anemia as result of repeated loss of blood from papillae that have been traumatized or gnawed away. After the first months such growths generally appear to be stationary, but in most instances this is only because the dry tissue over them is continually worn or gnawed off: if they are covered with a bandage they rapidly increase in height. Yet they no longer spread. have followed the hemoglobin percentage and the number of circulating red and white cells, and have made differential counts on the blood of a group of domestic rabbits during the period of the papilloma's appearance and early enlargement, without finding any significant changes. Animals with large cutaneous growths capped by dead tissue usually have some polymorphonuclear leukocytosis, which the associated bacterial infection would lead one to expect. As previously mentioned the growths resulting from implantation in the viscera of favorable hosts ordinarily cause death within a few months at most. The rabbits lose weight gradually and die in extreme emaciation.

Rabbit papillomas developing on the skin as the result of virus SUMMARY AND COMMENT inoculation can be readily transferred to the inner organs of favorable hosts by implanting bits of the living tissue. The growths thus produced proliferate actively as a rule and frequently cause death. Often they are markedly invasive and destructive; and they tend to recur after excision. Bacterial infection may greatly enhance their malignancy. Accidental dissemination may occur during operation, and

<sup>&</sup>lt;sup>3</sup> Personal communication from Dr. Shope. See also Paper II.

distribution to the peritoneal surface has been repeatedly noted. There may be no cellular reaction whatever about the invading epithelium of interior growths, but usually some new formation of connective tissue takes place, its amount varying inversely with the rate of epithelial proliferation. An immediate reason exists for the inflammatory changes and scarring found beneath long-established skin papillomas, in the trauma and secondary infection to which the projecting, necrotizing masses have been subjected. In animals dying of progressively enlarging interior growths the skin papilloma may long have been stationary in size.

The growths appearing after the transfer of papillomatous tissue to the inner organs are due to the survival and multiplication of transplanted cells. However, the virus can be readily recovered from them, in the case of wild rabbits. No distinctive changes in the blood of the host have been found. The virus itself is highly specific for the epithelium of the skin, failing to act not only upon that of the other organs thus far tested but even upon embryonic skin.

The papilloma frequently penetrates into the blood and lymph vessels, especially at the edge of implantation growths. The intravascular injection of fragments of it sometimes results in pulmonary nodules of characteristic morphology. These are due to survival and proliferation of the injected cells. Secondary nodules have been encountered at autopsy in a lymph gland and in the lungs, but under conditions more suggestive of operative dissemination of the growth than of true metastasis.

Implantation growths of the papilloma in favorable hosts have the morphology of epidermoid tumors of greater or less malignancy. They behave as these do and elicit similar changes in the surrounding tissue.

The attributes and potentialities of the papilloma will be further considered in Papers II and III.

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# All of the sections were stained with eosin and methylene blue.

Fig. 1. Growths resulting from the implantation into a wild rabbit (W.R. 20) of bits of the animal's own papilloma. The growth appeared on the abdominal skin 7 days after virus inoculation, and 5 days later some of it was shaved off and injected into the extensor muscles of both upper forelegs, the subcutaneous tissue of the right groin, and the right kidney, spleen, and liver (at several situations). The animal died 39 days later as result of the numerous growths. The inoculated leg muscles had been almost replaced, and so too with the spleen (A). An irregular nodule of considerable size (B) was found in the healed abdominal wall. It protruded into the peritoneal cavity, as did the kidney nodule, which lay mainly in the cortex (C). Several large masses can be seen in the liver. At D is the skin growth.

Fig. 2. The groin nodule and the mass in the right leg, halved. The growth in the muscle is multicentric, with an absence of gross encapsulation (arrow). The groin nodule is partly melanotic. Natural size.

Fig. 3. Surface appearance and cross-section of growths in the liver of a wild

rabbit (W.R. 18) killed 49 days after implantation of the papilloma into several of the abdominal viscera,—photograph taken to show the projecting masses and the pronounced, localized melanosis. Natural size.

Fig. 4. Implantation growth in the kidney of the same rabbit. Natural size. FIG. 5. Growth mushrooming into the abdominal cavity of the animal, a result of accidental dissemination in the laparotomy wound. The arrow points to the

growth in the abdominal wall. The mass like a glans penis projected from it into the peritoneal cavity. Natural size.

Fig. 6. Papillomatous nodules in the upper lobe of the right lung of a domestic rabbit (D.R. 1-22) that died 108 days after skin inoculation, and 94 days after implantation with bits of its own skin growth (shaved off 18 days after virus inoculation) into liver, kidney, stomach, muscles of forelegs, and subcutaneous tissue. The lung growths probably resulted from an accidental introduction of papilloma cells into the blood at the time of liver implantation. (For additional details of history, see text, page 716.) Natural size.

Fig. 7. Edge of an implantation growth in the kidney cortex of a wild rabbit (W.R. 20), the same animal providing Figs. 1 and 2. There was some round-cell reaction about this growth. × 160.

Fig. 8. Edge of a growth in the liver of a domestic rabbit. On the left the advancing epithelium is completely unencapsulated. The animal (D.R. 1-23) was

killed because moribund with "snuffles" 26 days after implantation with its own growth into some of the abdominal viscera. The grafts had grown rapidly. (See Fig. 15.) × 68.

Fig. 9. Part of a growth in the spleen of the wild rabbit of Figs. 1 and 7 (W.R. 20), showing an unusual number of mitoses, many of which are abnormal.  $\times$  550.

Fig. 10. Unusual type of growth in a domestic rabbit (D.R. 1-22—see legend of Fig. 6 for history). Many but not all of the implantation growths (Fig. 17) showed this structure. The section came from a large subcutaneous mass in the axilla, containing numerous papillomatous processes in good condition, separated by rifts containing fluid, a rare finding. × 82.

#### PLATE 38

- Fig. 11. Specimen from wild rabbit No. 18 (of Figs. 3, 4, and 5), to show the direct replacement of liver cords by an invading melanotic growth. There is no histological evidence of pressure by the enlarging mass. The melanin can be seen to one side of the nucleus of the differentiating cells. × 220.
- Fig. 12. Lower magnification of the edge of the growth furnishing Fig. 9. The advancing epithelial tissue is wholly unencapsulated and actively invasive. Numerous mitoses are visible. × 130.
  - Fig. 13. Lung nodule from a domestic rabbit (D.R. 1-64) killed 13 days after the intravenous injection of bits of its own skin papilloma. The alveolar tissue about the enlarging growth has been stretched and pressed together, giving the appearance of encapsulation.  $\times$  42.
  - Fig. 14. Invading margin of another lung growth from the same animal, to show the absence of encapsulation or other reaction about the invading, differentiating epithelium. Pressure has compacted the walls of the alveoli next the growth into a spurious capsule.  $\times$  312.

#### PLATE 39

- Fig. 15. Edge of an accidental implant on the parietal peritoneum of a domestic rabbit (D.R. 1-23),—to show direct invasion of a blood vessel by the cells of the growth. A platelet clot at one side of the vessel covers the cells completely but elsewhere it is patent. Serial sections demonstrated that the invading cells were at the tip of a slender epithelial process extending from the parent growth, a small portion of which, including a mitotic figure, is present at the upper, right hand corner of the picture.  $\times$  500.
- Fig. 16. Invasion of a lymphatic by an implantation growth in the leg of a domestic rabbit (D.R. 1-22—for history see text, page 716). The epithelial cells are much degenerated.  $\times$  250.
- Fig. 17. Part of a secondary nodule developing in an axillary lymph node of the same rabbit. The epithelium replacing the lymphatic tissue has keratinized along one side of some of the advancing processes. The growth is unencapsulated.  $\times$  75.

#### PLATE 40

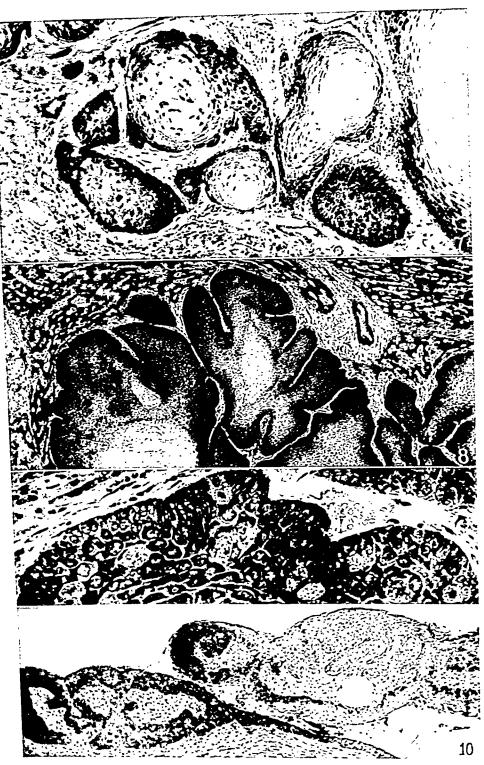
Fig. 18. Multicentric growth in the leg muscle of a wild rabbit (W.R. 20—see Fig. 1 for history). × 12.

Figs. 19, 20, and 21. Direct invasion and replacement of leg muscle by an implantation growth in a domestic rabbit (D.R. 1-20): section taken from the border of the growth. There is a cellulitis (Figs. 19 and 20) due to bacteria introduced with the implant. The animal died of general peritonitis 23 days after implantation in the muscles, liver, and kidney. Rapid enlargement of the grafts in the muscles had taken place, and so too with most of those in the viscera.

In Fig. 20 the epithelium is growing between and around the individual muscle fibres. In Fig. 21 it is directly replacing a fibre by proliferation within it. The sections all came from the periphery of a nodule that had extended far beyond the original site of inoculation.  $\times$  75,  $\times$  95, and  $\times$  270 respectively.



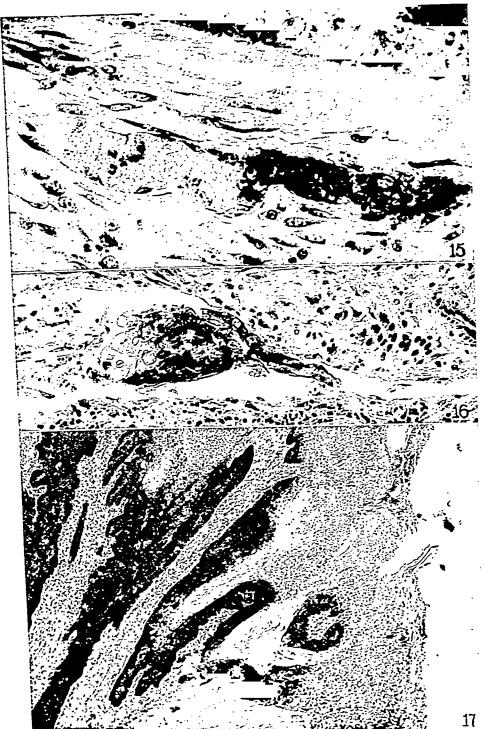
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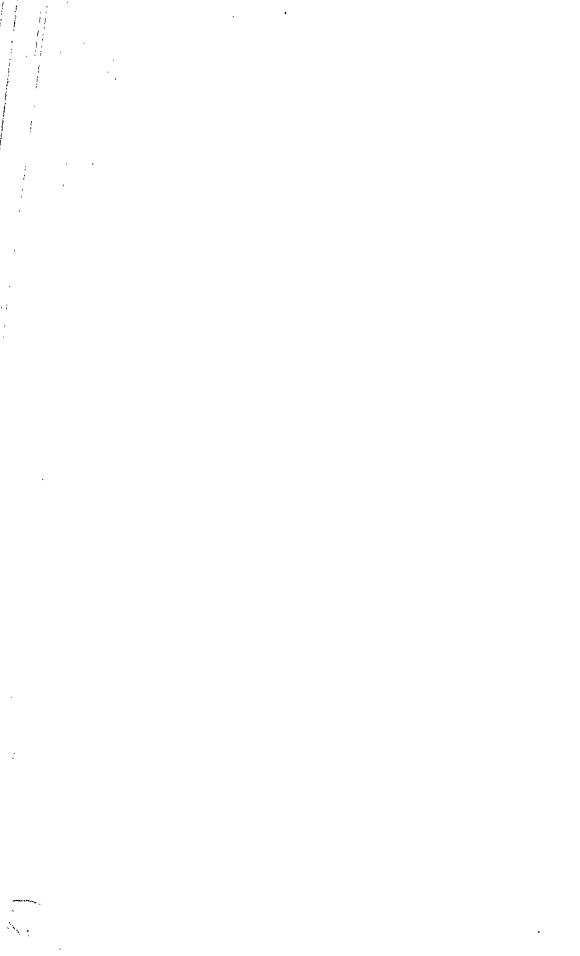








Photographed by Look Salantin



#### A VIRUS-INDUCED MAMMALIAN GROWTH WITH THE CHARACTERS OF A TUMOR (THE SHOPE RABBIT PAPILLOMA)

II. Experimental Alterations of the Growth on the Skin:

Morphological Considerations: the Phenomena

of Retrogression

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PLATES 41 TO 44

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The effects on the papilloma of transfer from the skin to the interior of the host have been described in Paper I. The influence of some chemical and physical agencies upon cutaneous growths will now be considered.

#### Stimulation with Scharlach R

The papilloma when implanted within the host grows progressively, invading and replacing normal tissues and killing the animal. Yet on the skin, its natural habitat, it invariably ceases to extend after a time, though its cells continue to proliferate, heaping up its mass. An observation recorded in Paper I suggests that this secondary limitation of growth is not due to the loss of an essential power on the part of the cells but to local conditions. Where incisions had been made tangential to established, stationary papillomas the latter grew into and for some little distance along the newly healed wounds, though elsewhere their borders remained unchanged. We have made numerous attempts to obtain aggressive skin growths by experimental means.

The subepidermal injection of a saturated solution of Scharlach R or of Sudan III in olive oil causes the epithelium of the rabbit's ear to burrow downwards and exhibit for a brief period the histological appearance and invasive tendency of carcinomatous tissue (1). Werner found the growth of a transplantable mouse carcinoma to be

greatly stimulated by local injections of the dye (2). true of the rabbit papilloma. IIThe same holds

Repeated injections of Scharlach R into the tissue immediately under and around the growth cause it to extend downwards and sideways instead of outwards, with result in fleshy, subepidermal masses (Fig. 22, B). The non-papillomatous epithelium next it partakes of the stimulating effect, but there the hair follicles are principally affected. Their epithelium grows downwards and they become distended, tending soon to round up into pearls lined with normal looking epidermis and filled with dead, concentric layers of keratinized cells (Fig. 23). The papilloma does not arise from the hair follicles but from the tissue between them (3); and its dye-stimulated epithelium retains a distinctive character, through becoming very irregular, thrusting long processes here and there into the tissue, and not infrequently entering the blood vessels. (See Figs. 15 and 16 of Paper I for vascular invasion in the absence of dye stimulation.) If Scharlach R is injected at one side only of a papilloma, the latter extends in this direction. Its effect upon old stationary growths can be exemplified by the findings in a domestic rabbit carrying, as result of broadcast inoculation on the abdomen, a large "pancake" of papillomatous tissue that had ceased to extend, and several smaller growths along one side where the virus had been tattooed into the skin. Repeated injections of the dye were made about these latter, and some found its way down through the tissues to the border of the "pancake." As result the mass underwent a pronounced secondary thickening, due to epithelial penetration into an underlying reactive Connective tissue; but this took place only on the side where the dye was present.

The skin over the large, fleshy, subepidermal growths that form as result of repeated dye injections under and around developing papillomas, tends to break down after a time, owing to pressure necrosis; infection supervenes; and the mass becomes foul and sloughing. But at the periphery, where Scharlach R still is present, an active proliferation and subepidermal extension continues.

No enduring increase in the activity of the papilloma has been brought about by Scharlach R: as the latter disappeared the growth became quiescent. And no certain instance of induced metastasis formation has resulted from its use, though a possible one is reported in Paper I. Repeated injection of the dye causes papillomas located on the ears of domestic rabbits to become huge, conical, cutaneous horns, as much as 5 or 6 cm. high, with fleshy bases and dry, brown, vertically striated peaks. The proliferating epithelium under the middle of one such horn (Fig. 25) penetrated to the under surface of the ear (Fig. 26) through a pre-existing lacuna in the cartilage, and formed a secondary nodule there (Fig. 27). This happened after

the coloration indicating the presence of Scharlach R had long since disappeared from the tissue. One may suppose that the epithelium took the way of least resistance from its confinement beneath the horny mass. This supposition seems the more reasonable because experiments that will now be described have shown that when a skin papilloma is prevented from extending outwards it will burrow into the tissue under it.

The Consequences of Preventing Outward Growth of the Skin Papilloma

To determine the effect of altering the mechanical conditions of growth a number of papillomas were covered with collodion.

It proved essential to apply the collodion when the first papule or papules appeared after virus inoculation, for if it was done later they either pushed out between the layer and the skin, like a flat, many petalled chrysanthemum, or when they tended to broaden at the base, they undermined the adjacent normal epithelium, killing it by cutting off its blood supply, with result in a broad ulcer with thick, fleshy edges. If, however the collodion was put on when the first punctate roughening of the epidermis was noticeable after intradermal injection of the virus, and if the layer was flexible and was not soon loosened by desquamation and growth of the hair, as too often happened, the epithelium affected by the virus, instead of projecting in elongated papillae, continued its primary extension into the connective tissue, and formed in the course of some weeks a thick, discoid mass between the fibrous layer of corium and the epidermis (Fig. 22, C). Often several centimeters in diameter, it continued to enlarge after the collodion had been stripped away. The epidermis over the center of such masses became tense and after a time broke down, but elsewhere it was only stretched and thinned. Like the implantation growths described in Paper I, they consisted of a rind of translucent. grayish pink tissue, about a dense, tough, dry, creamy to sooty, necrotic material which separated more or less readily into papilliform processes when cut across. Under the microscope the rind was found to consist of proliferating epithelium and reactive connective tissue, in papillomatous arrangement, with processes everywhere pointing toward the growth's center (Fig. 24). Here pressure necrosis had occurred. A more or less well defined capsule was present, consisting of newly proliferated connective tissue with, as a rule, almost no lymphocytes and no polymorphonuclear cells. Into this new tissue the epithelium had pushed forth in more or less orderly, close-ranked processes, incorporating some of it as cores of the papillae in process of formation. Thus the growth enlarged by progressive invasion of its surroundings as well as by expansion.

The papillomas prevented by collodion from extending outwards proved not only capable of enlarging beneath the epidermis but

they did so at the expense of the normal tissues. Though their encroachment was orderly it took place in a way suggestive of malignancy, and it was progressive. The stimulation of Scharlach R rendered the papilloma highly irregular and invasive, and from its results one derived the impression that sustained malignancy would result from continued stimulation. Such stimulation has been accidentally provided in not a few implantation growths through the influence of contaminating bacteria (see Paper I).

## Variations Due to the Influence of the Host

The character of the papilloma is influenced not only by local conditions but by the individuality of the host. In some rabbits the growth retrogresses, a phenomenon which will be considered further on; and even in favorable hosts it varies considerably. The differences in gross aspect of the papillomas produced experimentally on the skins of wild and domestic rabbits have already been described. They would appear to be largely due to differences in the skin itself as a supporting fabric, that of wild individuals being thin and rather dry whereas in domestic ones it is often thick and succulent. When it is thin in the latter the growths tend to resemble those in wild rabbits. Some differences observed in the response of the normal skin to injections with Scharlach R in olive oil are not without significance in this Thick, succulent skins thicken still further and their epidermis proliferates markedly, taking on an appearance like shagreen and later becoming scurfy; thin skins on the other hand appear almost unaffected. The papilloma is most fleshy and exuberant in precisely those skins which respond most pronouncedly to Scharlach R. The maximum variations in hosts favorable to the growth are well illustrated by the findings in two litter mates inoculated shortly after

The rabbits, of gray-brown, domestic breed, were inoculated when 8 days old with the same specimen of virus fluid, by rubbing it into the scarified epidermis over a broad expanse on the abdomen. The growth appearing in one was low and confluent, and in its superficial portion dry, friable and sooty, brittle, and porous, like a mass of coke (Fig. 28). In the other little rabbit it consisted of numerous separate, brown, radish-shaped cutaneous horns, several centimeters high, vertically

striated, tough and somewhat elastic, with bulging, fleshy bases. As these broadened the skin was forced into folds to make room for them all (Fig. 29). They consisted, as sections showed, of very numerous, thin papillae, some of which were alive far up into the brown horns (Fig. 31). The living tissue of the low growth was almost entirely restricted to a basal layer with but poorly developed, infrequent and blunt, papillomatous processes (Fig. 30). Yet this growth, though apparently stationary at the time when both rabbits were killed, 111 days after inoculation, had at several points invaded the lymphatics beneath it.

There was no histological evidence that bacterial infection was responsible for the differences noted. The animal with the low, dry papilloma had somewhat the thinner skin and had not increased quite so rapidly in weight as the other.

The animals of this experiment seemed to be in much the same bodily state and their skins differed little in the gross. The pronounced contrast in the character of the papillomas may well have been due to differences in reactivity of the host tissues. If the epithelium stimulated by the virus is to assume the papillomatous form, the skin must provide for it a supporting fabric of connective tissue with the necessary vessels,—in short a stroma. The behavior and morphology of the growth cannot but depend largely on the extent to which this stroma is forthcoming. A like dependence exists in the case of many tumors.

#### Morphological Considerations

The influences which determine the differing forms that the papilloma assumes on the skin and in the interior of the animal can be discussed conveniently at this point.

Irrespective of where the growth is situated the division of its cells goes on most actively in the deeper layers of epithelium, lessening and ceasing in proportion as the maturation into keratinized elements takes place. The growth rate may be considered in terms of a cone with its apex corresponding to the level in the epithelium where proliferation wholly ceases. The differentiating, keratinizing cells beyond this apex do not desquamate but remain firmly attached, a peculiarity largely responsible for the height of skin papillomas. The result for fragments of the growth that have been placed in the inner organs is that the proliferating layer of epithelial cells, extending laterally as result of its multiplication, yet tethered by the keratinized mass, spreads around the latter, the base of the hypothetical cone broadening until it has formed a sphere. Doubtless this encircling and enclosing would take place in uninvasive growths even if the keratinized cells did tend to come loose. Its consequence is that rounded nodules develop from

the bits of papilloma implanted within the animal, living epithelium enclosing dead squamous tissue, the apparent reverse of the state of affairs on the skin, where the latter is the covering layer. The way in which the two forms of the papilloma are produced finds direct illustration in those cases in which cutaneous growths have been forced to extend beneath the surface by covering them with collodion (Fig. The conditions on the skin surface do not enable the proliferating layer of epithelium to enclose the overlying keratinized tissue: instead it buckles into papillae. A secondary outward protrusion, with buckling and more papillae, occurs as result of proliferation within the confines of the surface mass.

The epithelium of interior growths frequently sends out processes into the surrounding tissue, but these tend to become spherical later for the reasons just given. An extreme instance of the phenomenon can be observed at the surface of the globoid implantation nodules which sometimes practically replace the spleens of wild rabbits. Here numerous, blunt, papillary outgrowths of proliferating epithelium may exist side by side, covered and held together by a film of peritoneum only; yet they never thrust forth separately into the abdominal cavity with result in a frankly papillomatous mass, because inevitably under the conditions each alters toward the spherical. Only once have we found an arrangement like that on the skin, this where an implantation in the kidney gave rise to a growth lying partly on the surface of the renal pelvis, where it took the form of a long, slender horn of

It is plain that the organoid appearance of the papilloma is largely due to the interplay of two factors,—one a progressive lessening in the rate of proliferation as the epithelium differentiates, the other a failure of the keratinized cells to loosen and come away from those beneath; but the importance of these factors must not be overrated. Finding expression independently of them is an innate tendency of the cells affected by the virus to assume the papillomatous arrangement.

These are the findings in growths which behave in an orderly way. But as the photographs show, growths resulting from implantation have often the morphology of epidermoid carcinomas. Where the epithelium has become aggressive, invading tissues actively as happens in many cases, it is no longer constrained to surround the differentiated, dead cells nor is its multiplication conditioned by the need for a stroma. It grows where it will, irregularly. Though pearl formation is frequent (Figs. 7 and 12), so too is keratinization on one side of rapidly advancing epithelial processes (Fig. 17). At some parts of the border of growths that appear orderly the phenomena associated with malignancy can often be detected. The benign looking papilloma of Fig. 30, for example, had penetrated at several points into the lymphatics.

The epithelial cells increase rapidly by mitosis in favorable hosts, and those which are dividing attract attention by reason of their great size and pallor, a pronounced stippling of the cytoplasm with granules that stain deeply with methylene blue, and a sharply defined chromatin arrangement. In very rapidly enlarging, malignant growths the division figures may be highly irregular, asymmetric, and hyperchromatic (Fig. 9), and many of the cells die before division is completed or shortly afterwards.

A frequent complication in the pattern and development of interior growths comes about through a swelling of the necrotic material that they contain. The importance of this factor increases directly with the amount of included, dead material.

Large growths are always tense on palpation though usually elastic, and where unsupported by the surrounding tissue they often bulge abruptly (Fig. 5) and may even rupture (lung growths, mesenteric nodules), with result in a form like a bud (Fig. 32). The broken epithelial layer may grow around the extruded squamous material secondarily, or when in a favorable situation, as in the lungs, it may extend edgeways into the normal tissue (Fig. 14), proliferating so rapidly as to keep ahead of differentiation, and hence of the influence of attached, keratinized cells to cause rounding up. Under these circumstances it appears highly malignant.

Ordinarily the pressure exerted by the necrotic, central material does not suffice to rupture the nodule, but acts to flatten and thin its peripheral layer of living epithelium, especially where this finds no support. Such a state of affairs is well seen where kidney or liver growths project from the organs (Fig. 4), the living epithelium here being often reduced to a layer one or two cells thick, flattened, and with no hint of the papilliform arrangement that is present near by, where it is backed by parenchyma. The changes consequent on gradually increasing pressure of the necrotic material are often clearly discernible in the markings of the latter. Its central portion may show the papilliform structure everywhere on cross-section. evidencing the character of the early growth, while further out the dead, keratinized lavers have a concentric arrangement, especially where there has been little mechanical resistance to the expansion of the mass, as where it protrudes into the abdominal cavity. Here in extreme cases the epithelium becomes entirely necrotic because its blood supply has been cut off. Where it has merely been thinned and flattened as result of the interior pressure mitoses are rare and there is an orderly differentiation to the keratinized state (Fig. 33).

Watch has been kept for variations in the underlying type of the papilloma. A single example only has been found thus far. In a

domestic rabbit dying of implantation growths in the liver, kidney, stomach, axilla, and extensor muscles of the hind legs,—an especially favorable host as shown by the occurrence of nodules in a lymph gland (Fig. 17) and in the lungs,—not a few of the implants showed unusually high and crowded, columnar basal cells, with processes extending into the connective tissue from the primary papillae at short intervals, rendering the growth a papilloma of the second order so to speak, with result in an unique appearance (Fig. 10). This state of affairs may have been the consequence merely of a stepping up of the proliferative activity of the basal epithelium.

### The Phenomena of Retrogression

Not infrequently an induced papilloma retrogresses. that it may do so in a distinctive way, perhaps indicative of its virus cause, has led us to study the process in detail. The possibility

Wild rabbits resistant on experimental inoculation with the virus are not infrequently encountered. Shope found none with complete immunity, though three which were carrying the growth when received proved partially resistant (4). We have found four with complete immunity against our virus amongst 25 inoculated, two of them having papillomas of "natural" origin, the only ones that had. Our animals came from the same source as Shope's. Those that showed themselves resistant have been inoculated repeatedly with active virus fluid, always ineffectually.

Gross Manifestations.—In an occasional cottontail the papilloma appears promptly and grows for several weeks, drying up and falling off later, leaving a smooth, healthy-looking skin. This has happened in several animals inoculated with virus procured from fresh material and of but slight pathogenic capabilities, as control inoculations on domestic rabbits showed. Thus far in our experience wild rabbits with visceral implantations of their own vigorously proliferating papilloma have regularly succumbed to the resulting growths, though the skin papillomas may have ceased to extend.

Adult domestic rabbits are without exception primarily susceptible to the virus, as Shope noted. Of the 64 thus far inoculated by us none has failed to develop a papilloma. The course of the latter has been found to vary greatly though. As already stated it grows best in

individuals with thick, succulent skin and may enlarge rapidly during the first week; but it regularly ceases to encroach later, remaining to all appearance stationary in most instances. Occasionally it undergoes retrogression. The mass dries everywhere, its base becomes thinner, and a gradual flaking away exposes healthy skin; or else the area of proliferating tissue beneath the mass dwindles gradually, with result in a tassel-like, pedunculated growth. The weight of this may act to elongate its pedicle (Fig. 34).

Retrogression occurs occasionally of one of several growths arising as result of implantations within a wild rabbit; but the process, in our experience at least, is restricted to nodules in unfavorable situa-This is the case as well in some domestic rabbits; but in others with papillomas caused by the same virus fluid the enlargement of all of the interior growths slackens after the first months and they assume the globular form (Figs. 36 and 37), becoming well encapsulated (Fig. 38) and notably firm. Complete necrosis of individual nodules sometimes occurs, especially of those in the subcutaneous tissue, kidney, stomach, and such as are precariously nourished from the mesentery or peritoneal lining through a pedicle (Fig. 36, P); but in no case thus far studied has the growth failed to progress elsewhere (Fig. 37) and kill the animal. It is reasonable to expect that instances of general retrogression will be encountered when the skin papilloma utilized for implantation has not been doing well. Thus far we have implanted only material from robust growths.

The Histology of Retrogression.—The retrogressive changes are best studied in sections from interior nodules, since here the complicating effects of trauma, drying, and infection can be excluded.

The influence of the pressure exerted by the necrotic material within large growths to cause localized retrogression at their periphery has already been mentioned. It should be discriminated from retrogression proper, and this can usually be done. Mention has also been made of cell death following upon abnormal mitosis. Necrosis may abruptly overtake individual groups of the epithelial cells of invasive, highly malignant growths, either because their own rapid proliferation has deprived them of nourishment or because they are not suited to survive. But none of these happenings constitutes retrogression in the proper sense. This takes two forms. The epi-

thelial cells may suddenly cease to proliferate, and all differentiate to the squamous form, their death occurring as result of maturation. Such retrogression is usually very local. Or there may be a more widespread retrogression which is gradual and follows a definite order. It resembles that brought about by pressure, as above described, save that there is no unusual flattening of the epithelial cells.

The first changes noted microscopically are a blunting and broadening of the processes at the border of the growth, and an encapsulation of it with dense connective tissue in which are a few round cells. In the gross one notes a rounding of the mass and a sharpening of its contours. Soon the epithelium no longer pushes out here and there, but becomes a smoothly curving rind about a necrotic mass which has the papillomatous arrangement at its center but is increasingly concentric toward the periphery. Mitosis becomes infrequent, the epithelial layer takes on an appearance of special orderliness, its cells and their nuclei become smaller and stain less deeply,—in a word the tissue reverts more or less completely to the normal in its appearance. Occasionally even where much thinned it may still have characters indicative of the presence of the virus, both cells and nuclei being abnormally large and staining darkly. As result of cell differentiation in the absence of multiplication the mass eventually comes to consist wholly of dead, keratinized elements. Where these first come in contact with the surrounding connective tissue some polymorphonuclear leukocytes may gather, followed later by granulation tissue and giant cells which, together with connective tissue elements, penetrate very slowly and ineffectually amidst the dead cell masses, breaking up the outer ones (Fig. 44).

Our method of implantation, with shavings from surface growths, must often have involved some introduction of normal epithelium with the pathological; and it might be supposed that survival of this, or a gradual replacement by it of the papillomatous epithelium, not true retrogression, was responsible for the thin, maturing layer of more or less normal looking epidermal cells found at the periphery of most stationary or dwindling nodules. In general, however, the pattern of the necrotic material at the center of such nodules proves that their growth was everywhere papillomatous at first; while furthermore each step in the retrogressive process has been studied without discovery of any replacement of the sort outlined. No hair follicles or sebaceous glands have been noted in association with the reordered, epithelial layer.

Surface growths in hosts developing resistance show few papillae, and these are usually short and necrotic nearly to their bases. The wide spaces between them contain dead, keratinized tissue save for a smooth, underlying layer of epithelium. In appearance this epithelium has reverted more or less to the normal, and its connection with the hair follicles and sebaceous glands is now once again evident, as was not the case when it was in active papillomatous proliferation. The underlying connective tissue is dense and contains some makrophages and scattered accumulations of lymphocytes. Where pedunculation of the growths is accentuated by their weight (Fig. 34) the infrequent papillae may remain alive even though much attenuated (Fig. 35). Their appearance and the general conditions suggest that they and their nourishing cores have been pulled out by gravity.

#### The Behavior of Growths on the Skin

In the skin of even the most favorable rabbits the extension of the papilloma ceases after a time. Though it enlarges rapidly at first,many scattered papules sometimes broadening and coalescing to form one mass, or a single papule developing into a cutaneous horn or warty growth several centimeters across,—its extension at the expense of its surroundings slows after some weeks and stops, though its cells continue to proliferate actively, the mass often becoming so redundant as to be thrown into thick folds and to lop over at the sides. Beneath it there is now a layer of dense connective tissue. Some of the epithelial processes which penetrate here and there into the latter are cut off by it from the surface and round out into pearls, often several millimeters in diameter, with a firm, yellowish white, spherical center of dead, concentric, squamous layers which shell out readily from a smooth, enclosing rind of living epithelium. Other processes not only maintain themselves but proliferate and burrow in irregular tongues amidst the dense connective tissue. Though the growth has ceased to enlarge, its appearance now suggests a developing malignancy. is always heavily contaminated with bacteria from the skin and one may suppose that, as in the case of infected muscle implants, these bacteria sometimes, by influencing the epithelial cells or by the changes they bring about in the connective tissue underlying the

growth, or by both combined, induce a more aggressive behavior. None of our papillomas has been followed for more than 6 months.1

The findings here reported, like those of Paper I, demonstrate the ability of the papilloma to grow progressively and destructively on favorable occasion. Why then when situated on the skin does it ordinarily cease to enlarge after a time? Its cells continue to multiply, but everywhere surrounding it a layer of dense connective tissue gradually forms. Our experiments have shown that the growth encroaches upon its surroundings before this appears, as also when it is cut through later, and when the new tissue does not have time to become dense before the epithelium pushes into it,—as in instances in which extension of the papilloma takes place beneath the surface as result of covering it with collodion. The new layer of connective tissue evidently holds in check the epithelial cells which, in the lack of special stimulation, have no considerable aggressive ability. Numerous observations attest to this last fact. When fragments of the papil-

<sup>1</sup> Dr. Shope has generously permitted us to study and report upon a skin papilloma from the abdomen of a domestic rabbit killed recently, 393 days after inoculation. The animal had previously recovered from a fibroma also induced by a virus (Shope, R. E., J. Exp. Mcd., 1932, 56, 803). The papilloma now under consideration had long ceased to extend and had been gnawed close, but there remained an indurated base nearly 1 cm. thick. Sections of this show an irregular, thin, surface layer of proliferating epithelium, manifesting the papillomatous arrangement in some places, and beneath it to a depth of 3 to 4 mm. irregular processes, cords, and groups of proliferating epithelial cells, amidst an edematous connective tissue in which the fibrillar bundles of the corium can be recognized, now largely fragmented. The epithelial cells show some differentiation to the squamous form, but are both invasive and infiltrative. The picture is that of an epidermoid carcinoma of unequivocal malignancy (Fig. 39). The regional lymph glands of the animal were not strikingly enlarged, nor were nodules seen in the lungs.

In this instance a secondary breaking up of the connective tissue barrier beneath the growth may have enabled it to penetrate deeply. Or conceivably an independent carcinomatous change may have supervened in epithelial tissue prepared by long standing injury referable to the virus. scarcely be advanced, however, to account for the prompt invasive and destructive growth from fragments of early skin papillomas implanted in the internal organs The latter explanation can of the host. The causative virus has been recovered repeatedly from the nodules developing as result of such implantation in wild rabbits.

lomatous tissue are distributed by the arterial blood, as in the experiments of Paper I, they give rise to growths in the lungs only; and when brought to the lungs by the venous blood they cause few nodules in comparison with the number of fragments introduced. The tattooing of individual papules that have just appeared on the skin after intradermal virus inoculation, as in some tests not mentioned heretofore, does not result in subepidermal growths from cells dislodged and driven under the surface, but instead the papilloma is wholly destroyed. Though the actively proliferating epithelium frequently enters the blood and lymph vessels, metastasis formation does not ordinarily ensue.

It is instructive to compare the findings with those on record for tumors. Many of the skin epitheliomata of man and of tarred rabbits and mice remain stationary and benign in appearance unless liberated by operation, or stimulated by infection, by renewed tarring, or in other ways. So too with some spontaneous chicken tumors. Many human carcinomata fail to cause metastases although cells from them are distributed in abundance by the blood and lymph (5). Not a few mouse tumors fail entirely to yield growths on intravascular and intraperitoneal injection of pieces of them,—in which respect the papilloma is relatively successful. There is a large literature on the restraining effects of the connective tissue associated with stationary and retrogressing epidermal tumors. Authorities hold it to be largely responsible for the behavior of the growths (6). Several instances are on record in which, according as the tissue barrier changed from time to time, becoming denser or thinner, the histological appearance of the tumor altered toward the benign or the malignant respectively (7).

In the phenomena of its retrogression the papilloma gives no hint of a virus cause. The gross and microscopic changes resemble those occurring in the case of epidermoid tumors. As with these, retrogression may be general or may take place at some situations while growth continues at others. In both instances individual cells or groups of cells may suddenly necrose as result of their own abnormality. Generally though when retrogressing the epithelium of the papilloma, like that of epidermoid carcinomas, ceases to proliferate and comes to its death as result of a process of orderly differentiation

into keratinized cells. This process occurs in man, though it is rare (8); it has been observed in spontaneous mouse tumors (9); and it is frequent in the case of the tar cancers of mice and rabbits. numerous published pictures of the changes in these last (10) show a detailed resemblance to those occurring in the papilloma. The retrogression of tar cancers, proven such by biopsy, is accompanied in many cases by a papillomatous rearrangement of the superficial masses, with reappearance of the connection of hair follicles and sebaceous glands with the epithelial layer, and eventually a complete reversion of the latter to the normal. A pedunculation may develop precisely like that illustrated in Fig. 34 of the present work. The retrogression of tar tumors that have penetrated beneath the surface leads to the formation of rounded, keratinized pearls lined by normallooking squamous epithelium. All of these alterations have been noted in the case of the papilloma.

The changes occurring in the tissue about retrogressing papillomas are like those about retrogressing tissues in general, irrespective of whether they are neoplastic. New connective tissue forms and lymphocytes accumulate in greater or less quantity. Where the dead, keratinized cells of epidermoid carcinomas first come in contact with their surroundings polymorphonuclear leukocytes may gather, to be succeeded by granulation tissue with giant cells, which slowly enters the dead mass (11). This is true of the papilloma as well (Fig. 44).

The injection of Scharlach R into the skin about rabbit papillomas resulting from virus inoculation causes them to invade the underlying tissue and form large, fleshy masses beneath the surface. cally these appear malignant, and they frequently invade the blood vessels. Covering young papillomas with a layer of collodion causes them to burrow downwards with result in discoid masses which enlarge progressively, both by expansive growth beneath the epidermis and by invasion. Such masses, like the nodules resulting from implantation, have the papillae turned toward their interior, the apparent reverse of the condition of affairs when the growth is situated on the skin surface. ties of the host influence skin papillomas not a little, as is plain from The reasons for this are analyzed. The peculiarithe forms they assume; but the epithelial changes induced by the virus take a single direction, and no significant variations from type have been encountered.

Local or generalized retrogression of the experimentally induced papilloma is not uncommon. The histological alterations that take place are identical with those attending retrogression of the epidermoid tumors, and the reactive changes taking place in the surrounding tissue are also like those about such tumors. The slowing and cessation of growth that occur secondarily in the case of virus-induced skin papillomas are associated with the formation under them of a dense layer of connective tissue, and to this their behavior is attributable. Similar findings have been often recorded for tumors, notably for the epidermoid cancers produced in rabbits by tarring.

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## EXPLANATION OF PLATES

### PLATE 41

- Fig. 22. The papilloma as influenced by physical and chemical factors. The virus was tattooed into the skin at four spots, each about 2 mm. across, on the side of a domestic rabbit (D.R. 1-22), and 22 days later when papules were just perceptible at sites A and B the first of several injections of Scharlach R around and beneath them was carried out. The growth did not appear at sites C and D until the 28th day. The papules at site C were covered with collodion on the 43rd day and those at D were left as control. On the 84th day the collodion was stripped away, with some petechial hemorrhage into the tense skin, as the photograph taken 24 hours later shows. The growths at A and B were for a long time almost completely subepidermal, ulcerating shortly before the picture was taken. They have coalesced. The discoid, subepidermal growth at C shows a crater containing dry tissue, the result of pressure necrosis. The control papilloma at D is much smaller than the others, wholly superficial, and with a somewhat constricted base.  $\times \frac{2}{5}$ .
- Fig. 23. To show the influence of Sudan III and Scharlach R. The growth was produced by a punctate inoculation of virus fluid into the ear of a domestic rabbit, and when a solitary papule had appeared, 33 days later, the tissue under and around it was injected with Sudan III in olive oil. Several similar injections of Scharlach R were made afterwards. There resulted a fleshy mass, largely subepidermal but ulcerating at its summit. The section, taken through its margin, shows irregular downgrowths of papillomatous epithelium with pearl formation. There are lacunae underneath, where the olive oil dissolved out. The epidermis covering the outer side of the mass shows some extension downwards and enlargement of the hair follicles, a characteristic effect of the dyes on normal skin. ×7.
- Fig. 24. Cross-section of a part of the growth under collodion shown in Fig. 22, C,—from a biopsy specimen removed on the 85th day. The growth lies in the corium. The living papilliform processes have their base at its periphery and extend into its necrotic interior.  $\times$  7.

### PLATE 42

- Fig. 25. Cutaneous horn developing from a solitary papule induced by punctate inoculation of the skin of the ear of an adult domestic rabbit (D.R. 9). Scharlach R in olive oil had been repeatedly injected beneath and around the papule soon after it appeared. The other papillomas on the ear can be disregarded, as pertaining to other experiments. The great conical horn was dry, brown, and vertically striated. Picture taken 96 days after inoculation and 63 days after the appearance of the growth.  $\times \frac{1}{2}$ .
- Fig. 26. A picture taken on the same day to show the extension to the under side of the ear.  $\times \frac{1}{2}$ .
- Fig. 27. Cross-section of the growth, made 7 days later when the ear was amputated. Most of the pale, papillomatous tissue is dead but still moist. Direct extension to the under side of the ear has taken place through a small natural opening in the cartilage, occupied in part by a vein and artery. Natural size.

Figs. 28 and 29. Differing character of the growths in two domestic, gray-brown litter mates. The skin of the abdomen was scarified and inoculated with virus fluid when they were 8 days old. The photographs were taken 97 days later, at which time both rabbits were in excellent condition and of nearly the same weight. The hair had been removed from about the confluent mass of Fig. 28 so that its low, dry, cindery character could be seen. The many separate cutaneous horns of the other (Fig. 29) were so large and crowded that a stretching and folding outwards of the skin had occurred to make room for them. The reductions are different: Fig. 28,  $\times \frac{1}{2}$ ; Fig. 29,  $\times \frac{2}{3}$ .

Figs. 30 and 31. Cross-sections of two discrete papillomas removed from the agglomerates of Figs. 28 and 29 respectively on the 70th day after inoculation. The differences in the lesions are described in the text. The tip of the growth of Fig. 31 was cut away to facilitate sectioning.  $\times$  7.

#### PLATE 43

Fig. 32. Periphery of an omental growth found in a wild rabbit (W.R. 18) killed 49 days after implantations into the abdominal viscera. The swelling of the necrotic material inside the nodule has caused it to rupture and "bud." The epithelium is flattened as result of pressure, and has reverted far toward the normal in appearance. Many of the other implants had grown actively (see Figs. 3, 4, and 5) and invaded their surroundings. × 125.

Fig. 33. Local retrogression, induced in part at least by pressure, at the periphery of a nodule in the liver of a wild rabbit. The animal was killed 48 days after implantation because the broad papilloma on the skin was growing smaller. The nodules that had developed in the muscles proved completely necrotic, but those in the abdominal viscera showed in general only localized regions of retrogression, with proliferation elsewhere in them. This was true of the growth furnishing the figure. In the part shown there is a pronounced flattening of the epithelium with reversion toward the normal.  $\times$  620.

Fig. 34. Ear of a domestic rabbit (D.R. 1-24), photographed 153 days after the virus had been tattooed into a number of spots across the tip, middle, and base. 5 days prior to inoculation some of the spots had been infiltrated with Scharlach R in olive oil (see Fig. 49). The growths became large and heavy, and their bases underwent a secondary constriction such that some of them came away. Only those at the middle and tip of the ear now remain, all notably pedunculated. The growths A, B, and C formed at one time a single mass, and A and B are still united by dead tissue though they have separate pedicles. Natural size.

Fig. 35. Portion of the base of papilloma C of Fig. 34,—to show the infrequent, attenuated, papilliform processes, and the exceptionally benign appearance of the growth.  $\times$  11.

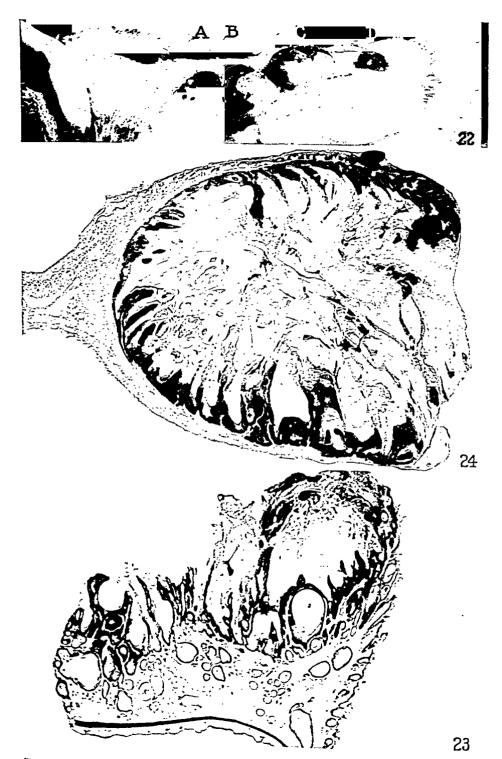
Fig. 36. Implantation growths in the abdominal organs of a domestic rabbit (D.R. 1-21) dying of them 133 days after skin inoculation and 115 days after implantation. There are large, coalescing masses in the liver, a projecting nodule (S) in the wall of the stomach, and others (P) on the omentum and mesentery,

resulting from accidental dissemination. Some resistance to the papilloma had developed (see comment on Fig. 37), and the nodules tend to be spherical and well encapsulated. At A is an implantation growth protruding from the capsule of the

Fig. 37. Growths resulting from implantations in the extensors of the upper forelegs of the domestic rabbit, D.R. 1-21. That in the right leg almost entirely enclosed the humerus and had caused toe-drop. Some resistance had developed in the host as shown by a falling off in the rate of enlargement of the masses, and by their smooth, rounded form and definite encapsulation.

Fig. 38. The mass in the right leg. It was encapsulated and necrotic save for a thin rind of the characteristic epithelium. Compare with Figs. 1 and 2, muscle growths in a favorable host.  $\times$  7/10.

Fig. 39. Section from the thick, gristly base of a growth that had endured more than a year. It was situated on the abdomen of a domestic rabbit killed 393 days after inoculation, and had been gnawed flat. Microscopically it had lost almost everywhere the papillomatous character and looked like an epidermoid carcinoma, as the picture shows. In the region photographed, which was several millimeters beneath the surface, proliferating epithelial strands and groups of cells have penetrated amidst a disorderly connective tissue which is dense in some portions, edematous in others. uninvaded tissue on the left of the photograph being its deepest portion. (Block The section was taken vertically to the surface, the



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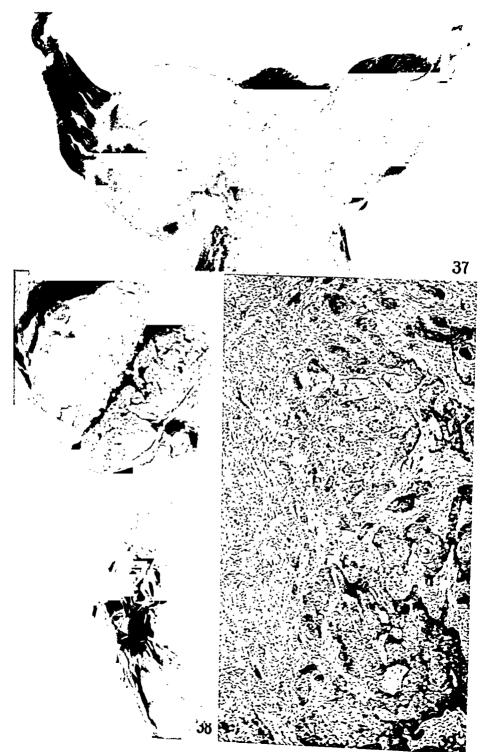


Photographed by Louis Schmillt

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Photographed by Louis Schmidt



## A VIRUS-INDUCED MAMMALIAN GROWTH WITH THE CHARACTERS OF A TUMOR (THE SHOPE RABBIT PAPILLOMA)

## III. FURTHER CHARACTERS OF THE GROWTH: GENERAL DISCUSSION

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PLATES 45 AND 46

(Received for publication, August 9, 1934)

In Papers I and II the behavior and appearance of the papilloma under various circumstances have been described. They are those characterizing the tumors. Yet the cause of the growth is a filterable virus. Are there no distinguishing signs of its presence? This question will now be taken up.

## Concerning Inclusion Bodies

The morphology of the growing or retrogressing papilloma does not suggest the presence of a virus. But in one important respect it has been insufficiently dealt with,—the possible presence within the affected epithelial cells of inclusion bodies such as occur in certain virus diseases has not been considered.

Hurst (1) could find no inclusions but we have renewed the search and have compared the cytological findings with those in some epidermoid carcinomas of man and tar cancers of mice. The sections of human tumors we owe to the kindness of several pathologists. A large variety of mouse tumors were available, deriving from previous work in this laboratory. The immediate point to be determined was not whether "bodies" can be discovered in the cytoplasm or nucleus of the papilloma cells but whether any exist that are not present in the recognized neoplasms as well. The sections of the papillomas and tar tumors were stained with methylene blue and eosin, or with Giemsa; those of the human epitheliomas in several ways.<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> The interpretation of the presence of inclusion bodies in tumors has of late become complicated with the recognition that these may not only harbor viruses

The literature on strange bodies in tumor cells is a teeming one, most of it dating back thirty years or more (2) to the time when some investigators thought that they could perceive an intracellular cancer parasite. It need not be reviewed. DeMonbreun and Goodpasture have recently summarized the findings on inclusion bodies in the warts and papillomas caused by viruses (3). It is remarkable for the diversity of the forms described, amphoteric, acidophilic or basophilic, cytoplasmic or intranuclear, sometimes occurring in certain regions only, or supposedly at special stages in the development of the growths. There has been no consistency in the findings.

In addition to the large eosinophilic inclusions, described by Hurst, which are the remains of dead cells, a variety of cytoplasmic bodies can be seen in some of the papillomas studied by us; but they are wholly lacking in others. They occur especially where the cells are differentiating into the granular layer, and within this layer, which is unusually rich in the granules that give it its name.

Sometimes the bodies take the form of pyknotic, crescentic masses such as Kyrle has described in warts, psoriasis, and other skin conditions (4). just outside one or both ends of the nucleus, as if extruded therefrom. Again they appear as evenly staining bodies, dark with methylene blue, filling the nucleus where keratinization is in progress, "like an egg within its shell" (Lipschütz (5)). Others are moderately refractile, rounded or oval, alkaline or amphoteric, and are first noted in the cytoplasm of cells immediately beneath the granular layer, reaching their greatest size in the latter (Fig. 40) and fading and disappearing at the same time as the nucleus, when the cells become keratinized and die. these various bodies are encountered in surface growths, being seldom seen in interior ones or in those that are retrogressing and orderly. They are most pronounced where the cells are unhealthy as result of infection or maceration, being found especially in the deep crypts between papillae, where the differentiating epithelium has undergone a localized swelling with hydropic enlargement of the nucleus. None of them resembles the distinctive virus inclusions, and all appear to have originated from ordinary cell constituents,—to be the outcome of excessive granulation and granular fusion, of nuclear pyknosis, or of localized cytoplasmic keratohyalinization. Similar bodies have been present in some of the epidermoid cancers of man and the mouse that we have examined, notably where there was a heavily granulated layer of differentiating neoplastic cells at the skin surface or just beneath it. Often they were present in the thickened, neighboring epidermis as well.

DeMonbreun and Goodpasture have described groups of large, clear epithelial cells in the tissue of a transmissible dog papilloma caused by a virus, which failed to differentiate with the rest of the layer in which they were situated (6). These they

<sup>(</sup>Levaditi and Nicolau (8), Rivers and Pearce (9)), but that the latter may cause within the tumor cells the inclusions characteristic of them (10).

regard as characteristic of the disease, reporting their presence in human warts also, where Lipschütz found cells having much the same appearance (7). No such elements are present in the Shope papilloma, but hydropic changes are frequent in unhealthy growths, as also in epidermoid tumors.

In sum we have failed to discover in the papilloma inclusion bodies such as indicate the presence of a virus, though various peculiar intracellular elements are to be found like those in the cells of the epidermoid tumors of man and the mouse. Certain characters to be noted in the papilloma when growing on the skin, namely great proliferative energy yet slight capacity for aggression, a thick layered and folded epithelium with an accentuated granular stage of differentiation, together suggest a virus cause, for the reason that they are present together in some of the warts, condylomas, and papillomas known to be due to such cause. Individually these characters are not distinctive, however, and their association is far less evident and frequently missing when the papilloma is growing inside the body.

## The Reaction about Beginning Papillomas

The study of precancerous conditions and of beginning cancers has shown that pronounced changes in the connective tissue usually precede and nearly always accompany the first appearance of the growth (11); and the suggestion has frequently been made that to them the latter is really due. It seemed conceivable that the first changes produced by the papilloma virus in and about the epithelium might differ in significant ways from those occurring in and about epidermoid tumors.

Virus was rubbed into broad scarified areas on the sides of a group of domestic rabbits, and each day thereafter, until the papillomatous roughening showed itself, a narrow strip of skin was removed from the etherized animal. Charts were kept of their location as a check upon whether they came from the midst of skin in which the growth subsequently developed. The sections were stained with eosin and methylene blue.

The scarification was found to have caused definite breaks through the epithelial layer, as a rule almost midway between the openings of the hair follicles. Within the next few days the layer reunited beneath a light scab of dried serum containing some red cells. This soon flaked off exposing a skin surface that in the gross appeared smooth and normally pale. But within 7 to 12 days after the inoculation a patchy reddening took place, and the strips removed at this time showed the

Shope noted that the intravenous inoculation of virus, while not giving rise ordinarily to growths, may induce them where the epidermis has been traumatized. It will do so as well where the skin has been damaged by Scharlach R, as the following test makes plain.

Scharlach R in olive oil (0.2 cc.) was injected at a number of points arranged in a circle about 7 cm. broad on each of the shaved sides of a domestic rabbit; and the injections were repeated after 18 days. 8 days later, when the local discoid thickenings were about 1 cm. across but stationary in size, or beginning to dwindle in some instances, 5 cc. of 5 per cent virus fluid was injected into an ear vein. Papillomas developed within a few weeks in seven of the fourteen thickenings on the right side and three of fifteen on the left. None appeared anywhere else. Certain of the reactive areas (Fig. 46), occurring at a time when the others were disappearing because the effects of the dye were wearing off.

The following experiment was done to find whether regenerating epithelium is especially susceptible to the virus.

Virus fluid was rubbed into large scarified areas on the abdomens of four domestic rabbits, and after 26 days, when the sharply defined "pancakes" of confluent papillomatous tissue were already 2 to 5 mm. high, two circular discs about 4 cm. broad were excised at their periphery, about half of each disc being normal skin and half papillomatous. The normal skin was cut through first, under ether anesthesia, and great care was taken not to sow fragments of papilloma on the raw surface. Under a dressing of sterile paraffin with a low melting point (Ambrine) clean healing took place, a smooth sheet of epithelium extending from both the normal and the papillomatous sides. But within a few days that from the latter source became rugose and developed abundant papillae, the result being that soon the old contour of the growth was approximately restored, as successive tracings There was never any creeping of the papillomatous condition around the margin of the healing disc, such as might have resulted from a spread of the virus infection by continuity to the regenerating cells, though at a few places where the papillomatous tissue was exuberant the new epithelium growing out from it upon the denuded surface advanced more rapidly than did the normal epithelium and covered a larger area eventually. With the development of papillae a sharp demarcation became evident, the new papillomatous tissue rising cliff-like next the regenerated, normal epidermis to which it was joined.

No signs were encountered in this experiment of enlargement of the growths by contact infection of the regenerating epidermis. But their absence might be accounted for by the change the virus undergoes in the tissue of domestic rabbits which renders it incapable in

most instances of causing the growth anew. This explanation will not cover the results of the tests now to be described.

A circular disc of skin about 4 cm. across was cut from each side of the abdomen of three etherized, domestic rabbits, and the raw surface was dressed with paraffin which was replaced at intervals. After 12 days, when the raw surfaces were just covered with new, thin epithelium, the whole surface and the adjacent normal skin were flooded with virus fluid, and through it two strips about 2 mm. wide were tattooed at right angles to each other, from normal skin to normal skin through the middle of the healing area, the result being a cross of skin punctures, each arm of the cross from 2½ to 3 cm. long. The fluid was now blotted off and a dressing put on as before. When the discs were excised India ink had been tattooed into the skin at four equidistant points about 0.5 cm. outside them; and the dark spots now lay just beyond the ends of each cross, serving to show its situation. After 2 weeks the papilloma began to appear, and it soon assumed the exact shape of the cross, being restricted at first to the tattooed strips. It increased somewhat more rapidly in height in the newly covered areas, as would follow from the more abundant vascularization, but did not appear sooner or broaden more rapidly there.

In this test the papilloma appeared only where virus had been directly introduced into the old and new epidermis. No spread occurred in the new-formed epithelium to indicate that it was especially susceptible.

In the large number of wild and domestic rabbits thus far observed by us, some of them carrying the papilloma at many situations, no secondary growths have appeared under circumstances indicative of spontaneous infection with virus from an existing focus. Enlargement never occurred by saltation, by an appearance of separate small papillomata near the growing edge.

The surface growths of wild and domestic rabbits have been incised on many occasions, and on others implantation nodules have been removed from the muscles. The experiments sometimes involved large skin incisions. During the implantations (Paper I) no pains were taken to prevent the suspension of tissue fragments from coming in contact with the traumatized epidermis. That it must often have done so was indicated by the frequent development of implantation nodules in the healed wound. Yet only once did a growth appear on the epidermis, and then as a solitary small papilloma after laparotomy, occurring under circumstances which made implantation probable (Paper I).

Numerous attempts were made to induce malignancy in both wild and domestic rabbits by repeatedly infiltrating the base of the papilloma and the surrounding tissue with Scharlach R (Paper II). The dye injections were begun when the

growth was not more than 1 or 2 mm. across, and the injecting needle was thrust into the neighboring skin at several points, thus adding trauma to the effects of the The latter sometimes caused a more or less widespread, shagreen-like thickening of the skin for a centimeter or more about the papilloma, with epithelial downgrowths of the well known sort. Yet though tattooing the virus itself directly into skin thus changed resulted in "takes" that were often extensive, in animals previously free from the papilloma,—no secondary growths ever developed about existing ones. True, the papilloma grew rapidly as result of the dye stimulation, but in the main by proliferation beneath the surface (Paper II).

As a whole the experiments demonstrated the following points. The virus acts only upon cells that have been rendered susceptible in some way; intradermal injections of Scharlach R, as well as trauma, will bring about the susceptible state; the virus can localize out of the blood stream upon susceptible cells; and when introduced directly into skin containing such cells it may undergo some local distribution. Such are the findings with normal animals and virus fluid obtained by the grinding and extraction of papillomatous tissue. Significantly different are those when the source of the virus is a papilloma in situ. No evidence has been obtained of secondary infection with the virus from an existing growth, even when the adjacent epidermis has been rendered susceptible. In regenerating epithelium no extension of the papillomatous change takes place suggestive of

Shope proved that animals carrying the papilloma become resistant to secondary inoculation with the virus and have neutralizing antibodies in circulation (12). These were demonstrable within 14 days after inoculation and 6 days after the growth appeared. They may prevent the transfer of infection from an existing papilloma to the adjoining cells. The further possibility exists that under natural conditions the virus is confined to the affected cells.

# Histological Relations to the Surrounding Epithelium

The best opportunity for the virus associated with a papilloma to ffect the neighboring epidermis may come in the early days, beore antibodies have been induced. We have examined sections of any early papillomas with this possibility in mind. Their proliferatg epithelium grades into that of the normal epidermis rather abptly, as Hurst observed of older growths, yet still so gradually that

it is impossible as a rule to say where one leaves off and the other begins (Fig. 41). So too where the growth is surrounded by an epidermis that is thickened and proliferating as result of the influence of Scharlach R (Fig. 23). No foci of papillomatous change can be perceived in the tissue thus altered. Occasionally when established papillomas are pigmented, or their cells are especially crowded, a fairly sharp histological distinction does exist between the virus-affected epithelium and the normal layer continuous with it (Fig. 47). In other, more frequent, instances the connection with the surrounding epidermis is accidentally torn through or macerated, and the enlarging growth is isolated by a cleft (Fig. 48). In yet other cases the papilloma undermines the epithelium next it, with result in a necrosis that destroys the continuity of the two, and henceforth it proliferates independently.

All these various relations are found at the edges of the epitheliomata of mice and men. The histological evidence for a conversion of normal to papillomatous epithelium at the edge of the papilloma is neither less nor greater than in the case of these neoplasms. Such conversion was greatly debated amongst the older students of cancer. Ribbert, the main advocate of the view which now prevails, that tumors once well started grow only by their own intrinsic proliferation,—aus sich heraus.—himself produced evidence that epidermoid cancers may begin at several neighboring centers (13), a fact now generally conceded, as result more especially of the study of tar tumors. He pictured epitheliomata that were directly connected with the epidermis, grading into it by a gradual transition; others in which, though the epithelial layers of the two were continuous, pronounced histological differences distinguished them; and yet others in which a secondary break had occurred to separate them.2 In no feature does the papilloma more closely resemble the classical epitheliomata than in these

<sup>&</sup>lt;sup>2</sup> That a connection between two widely differing sorts of epithelium may come about secondarily has been shown by experiments in which cell groups of the Flexner-Jobling adenocarcinoma of the rat united with regenerating epidermis (Rous, Peyton, J. Exp. Med., 1913, 17, 494). In the course of the present work we have observed instances in which the papillomatous epithelium of implantation growths became directly connected with the epithelium of the kidney pelvis (Fig. 49), with that of the mammary duct, and with the peritoneal lining.

relations to the neighboring epithelium. To all appearance once the growth has been started it enlarges by its own proliferation. Our experimental observations, recorded a few pages back, provide reasons for the supposition that in general it really does so.

## Attempts at Transplantation to Other Individuals

The presence in the papillomatous tissue of a causative virus almost uniformly infective for rabbits lends special significance to the fate of this tissue on transplantation. Bits of it placed in the inner organs of the host grow with such regularity that one might suppose transplantation to other individuals would be readily accomplished.

Experiment 1.—In order to free the papillomatous tissue, so far as possible, from contaminating bacteria, a preliminary subcutaneous implantation was made in the axilla of a wild rabbit (W.R. 10) of bits shaved from a papilloma on the skin of the abdomen. There resulted a large growth. It was removed, under ether, 65 days after the original inoculation with virus and 24 after implantation; the living epithelial rind was cut up; and part was suspended in Tyrode solution and injected into the extensor muscles of the upper forelegs of the donor, three other wild rabbits, and three domestic ones. With the remainder 5 per cent virus fluid was made in the usual way and centrifuged; and some was withdrawn from the center of the column of fluid and heated at 53°C. for 15 minutes to kill any tissue cells present. It was then rubbed into a large, freshly scarified area on the lower side of W.R. 10 and into areas of similar size on the abdomen of the other animals. Sections proved that the tissue utilized had been actively proliferating.

All of the rabbits except W.R. 10 developed papillomas as result of the skin inoculations with virus, this individual showing itself completely resistant. The growths thus caused retrogressed in one of the wild rabbits; and in one of the domestic species they were discrete, though numerous and rapidly growing. The other four animals promptly developed confluent "pancakes" of papillomatous tissue. Only in W.R. 10, however, did growths result from the tissue implantations in the forelegs. They appeared within 10 days in this individual, and when it was sacrificed, 79 days after implantation, they measured 3 cm. and 2.5 cm. in diameter respectively, and had the characteristic make up. The rabbits were kept under observation for months. In some of them nodules 2 to 4 mm. in diameter soon appeared at the sites of transplantation but after several weeks could no longer be felt.

The rabbit providing the tissue for transplantation proved insusceptible to infection with the virus extracted from this tissue, yet

<sup>&</sup>lt;sup>3</sup> Shope has shown that the virus in 5 per cent suspension is unaffected by heating for 30 minutes at 65°C.

favorable to implantation with the cells. The other rabbits showed themselves susceptible to the virus though the tissue providing it failed to grow in them.

Experiment 2.—The general procedure was the same as in Experiment 1. The material for transplantation came from Domestic Rabbit 1-34 which had large implantation growths in all four legs. One was removed piecemeal, by operation under ether, and minced and suspended in Tyrode. Part of the suspension was filtered through gauze and injected into an ear vein and artery of the host. The unfiltered remainder was injected into the extensors of the upper forelegs of three wild rabbits and three normal domestic ones, into another domestic rabbit recently recovered from vaccinia, and into one in which a previously induced skin papilloma had retrogressed. No muscle injections were made in D.R. 1-34. To rule out the possibility that the needle might carry some epidermal cells before it into the deeper tissue, infecting them with virus simultaneously and thus causing growths which were not transplants at all, a small slit was made in the skin at each implantation and its lips were held apart so that the entering needle did not come in contact with the epidermis. A 5 per cent extract of some of the papilloma tissue was prepared in the usual way, heated at 53°C. for 15 minutes after centrifugation. and rubbed into the scarified abdomen of all of the rabbits.

The donor, D.R. 1-34, died of intercurrent causes 14 days after the intravascular inoculation,—which had produced no visible growths. It carried a large, foul abdominal "pancake," and implantation nodules up to 3 cm. in diameter in all of the legs except that furnishing the material for transplantation, which showed small recurrences in the subcutaneous tissue of the healed incision. The virus inoculations failed to cause the papilloma in any animal, and the transplantations resulted merely in transient, small nodules in some of them. One such nodule, taken from a domestic rabbit that died of intercurrent illness 7 days after the transplantation, contained proliferating and well vascularized epithelium of the characteristic sort (Fig. 42), surrounded by new connective tissue in which were a few lymphocytes. At several points epithelial processes were extending out.

In one of the wild rabbits receiving implants, which died of intercurrent causes 37 days later, sections showed the transplanted tissue to be surrounded by an accumulation of small round cells so dense as to have the superficial appearance of a lymph node (Fig. 43). The tissue was now necrotic, but the shape of its largest fragment showed that it had undergone some early proliferation. The lymphocytic reaction was far more pronounced than that about dead autoimplants in domestic rabbits (Fig. 44).

The papillomatous tissue utilized in this experiment had been actively invading the muscle, as sections showed. That it was capable of surviving on transplantation to another individual was proven by the findings (Fig. 42) in the domestic rabbit that died early; yet it

failed to cause growths in any of the surviving animals except the host, in which nodules developed as result of accidental dissemination when the transplantation material was procured. The wholly negative results of the virus inoculations accord with Shope's experience that neither wild nor domestic rabbits can be infected ordinarily with an extract made from the papilloma of a domestic animal.

Experiment 3.—A wild rabbit (W.R. 18) with a papillomatous "pancake" and rapidly enlarging implantation nodules in the foreleg muscles, right kidney, liver, spleen, stomach, and groin, was killed and material taken from the surface growth. This was large and high with a dry, tough, fibrous, outer portion which was sliced off separately, and extracted in the usual way, yielding what may be termed Virus Fluid I. The base of the growth was similarly treated to procure Virus Fluid II, the intermediate part in which dead and living epithelium were mixed being discarded. Portions of the living rind of the leg nodule that had enlarged most rapidly were extracted for Virus Fluid III, and other portions of it were added for transplantation purposes to parts of a nodule found on the splenic omentum, and one from the parietal peritoneum. The pooled material was minced, suspended, and injected into the upper forelegs of three normal wild rabbits, three in which a previous skin inoculation with virus had failed, and three normal domestic animals. The skin was slit just beforehand, as in Experiment 2. virus extracts (5 per cent in each case, unheated) were rubbed into scarified areas on the sides and abdomen respectively of three other normal domestic rabbits and three wild ones, the site being varied for each extract.

The animal furnishing the experimental materials had large, actively growing nodules at several implantation sites in the viscera, and one of these (from the splenic mesentery) yielded a virus of unusual activity after some weeks glycerinization, as animal tests proved.

The transplantations resulted in nodules as much as 4 mm. across in some instances, but all had disappeared within 3 weeks, though the microscope showed that vigorously growing material had been implanted. No attempt had been made to prevent the suspension fluid from coming in contact with the slit skin when the needle was withdrawn, and in one of the domestic rabbits a papilloma developed in the linear scar. Virus Fluid I gave rise to skin papillomas in two of the wild rabbits and one domestic; Fluid II caused them in these same wild rabbits and the two other domestic ones; while Fluid III produced the papilloma only in one of these last. Although skin areas about 5 cm. across had been scarified and inoculated broadcast, discrete, slowly growing, cone-shaped growths appeared which were obviously the result of scattered, punctate infection. In several of the rabbits they retrogressed.

In this experiment, like the others, the transplantations failed to

yield growths. Five of the six rabbits of the group inoculated only with virus developed papillomas in consequence.

Prior to the efforts at transplantation it had been supposed that the papillomatous tissue might grow on transfer to rabbits that were resistant to the action of the causative virus as such; for animals with flourishing growths frequently prove refractory when reinoculated with the virus,—a fact exemplified in W. R. 10 of Experiment 1. To test the point in Experiment 3 grafts were placed in several proven immunes to the virus, but with negative results. It had been further assumed that the virus would "take" when grafts could scarcely be expected to do so, for example in domestic animals receiving papillomatous tissue from cottontails; and for this reason transplantations to some rabbits of alien species, together with virus inoculations, were included in Experiments 1 and 2. The results justified the assumption, the virus producing growths in individuals unfavorable to transplantation (Experiment 1). It seemed conceivable that the inoculations with virus might affect the outcome of implantations made simultaneously. For this reason a separate group of animals was employed for the inoculations of Experiment 3.

The total number of rabbits implanted with papillomatous tissue deriving from other individuals of the same species was only fourteen, nine of these previously normal, three recently refractory to the virus as such, one immune to vaccinia, and one in which a previous papilloma had retrogressed and disappeared. In the one normal animal that died early the implanted material had survived and proliferated slightly. No extensive test this; yet since it has shown that the papillomatous tissue can live for a little while at least in the new host, there is a possibility that transplantation into rabbits of a single strain, or into a large number picked at random, may yield transplantable growths.

The evidence is convincing that the fate of the papilloma on transplantation is not determined by the presence of the virus associated with the tissue and inducing its proliferation. This virus as such will "take" vigorously, causing an exuberant papilloma, on the skin of individuals unfavorable to implants of the tissue with which it is associated (Experiment 1). This tissue, though failing to grow on transplantation to other individuals, will proliferate rapidly when grafted in the host, though the latter is now insusceptible to the direct action of the contained virus (Experiment 1). It is plain that two

sorts of resistance, occurring independently of each other, may be manifested by individuals to which pieces of the growth are transferred, these being directed against the implanted cells and the agent causing their proliferation, respectively. Such a state of affairs has already been demonstrated for the chicken tumors (14).

The fate of engrafted tumor cells is known to be determined by the laws of compatibility which influence the fate of transplanted tissues generally. This is evidently true of engrafted papilloma cells as well. Though the proliferative activity of the growth is great, it is possessed of but slight capacity for aggression (Paper II),—a fact which probably has some subsidiary influence on the outcome of transplantation. But many spontaneous mouse tumors that are highly malignant for the host and can be readily grafted within its body fail to grow on transfer to other individuals picked at random.

The cellular changes about implants that survive or die respectively would seem from the scanty evidence at hand to be identical with those about tumor grafts,—which is scarcely surprising since the reactions about the latter are elicited by them as tissue not as tumor.

## Influence of Other Disease Processes, and of the Host's Age and Condition

Certain additional observations and experiments deserve record, though unconnected with the immediate purposes of this report.

Not only does the virus fail to affect embryonic epithelium (Paper I) but the skin of new-born rabbits appears to be relatively refractory to it.

Two litters from gray-brown, domestic does were inoculated a few days after birth by rubbing virus fluid into the scarified skin of the abdomen, and in some instances injecting the skin of the ears. The does were similarly treated and some other domestic rabbits as well. All the adults promptly developed vigorous growths. In the five young rabbits on the other hand the papilloma appeared much later and at relatively few points.

The rabbit pox (15), a virus disease having many similarities to vaccinia, was epidemic in our animal room during part of the period of experimentation, and many of the animals bearing the papilloma, or in which it was just appearing, showed pocking with the "snuffles" and conjunctivitis that accompany the malady in its pronounced form.

Some became seriously ill; but in none was any effect manifest on the papilloma. To test the influence of vaccinia a group of domestic rabbits were injected intradermally at several points on the sides with the New York Board of Health strain, as propagated in tissue cultures by Dr. Rivers, at a time when the papilloma was just appearing in them and in an appropriate control group, as result of virus inunction into the skin of the abdomen. Large vaccinial lesions developed, but the disease had no evident effect upon the development of the papilloma. In studying the growth we have of late used many domestic rabbits recently recovered from experimental vaccinia. The findings were regularly the same as in the normal animals employed with them.

Many spontaneous and transplanted tumors of mice and rats are adversely affected by ill health or loss of weight of the host, and this is the case with Chicken Tumor I as well. The Shope rabbit fibroma, a growth caused by a virus, is in our experience remarkably susceptible to such influences. The papilloma on the other hand is not notably affected either by malnutrition of the host or by intercurrent illness. But this is the case, for that matter, with not a few of the recognized mammalian tumors.

Shope could elicit no cross-immunity between the papilloma and his rabbit fibroma caused by a virus. A test with the Brown-Pearce rabbit tumor seemed desirable,—the more so since the cells of the latter growth are of epithelial nature.

Thirteen adult, domestic, gray-brown rabbits of the same size were procured, three of them set aside as controls, and ten inoculated intradermally with 0.2 cc. of Tyrode suspension of Brown-Pearce tumor at four sites on the back of the neck, as well as with 0.3 cc. intramuscularly in each hind leg. The object was to cause tumors which would undergo retrogression because in unfavorable situations. Within the succeeding 46 days this was the course of events in four animals, the others either developing progressive tumors or failing to show any. Most of the intramuscular growths that retrogressed were large at one time. Now the four animals were injected again intramuscularly with Brown-Pearce suspension, this time into the muscle of the forelegs, as were the controls, and on the same day virus fluid prepared as usual from glycerinated papilloma tissue was rubbed into the scarified abdominal skin of them all. Intradermal titrations of the papilloma virus were not employed because of the uncertainty of the method and the long period elapsing prior to the appearance of growths,—a period during which

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induced resistance to the Brown-Pearce tumor might have fallen off. The local variations in the conditions of virus inunction upon the broad areas that were scarified may have served as a rough substitute for titration, the papilloma appearing late and at scattered points in some parts of such areas, whereas at others a confluent papillomatous change took place early.

The rabbits in which the Brown-Pearce tumor had retrogressed did not develop tumors from the material of the second injection whereas the controls did so, the growths in two of them enlarging rapidly. The papilloma appeared in every animal, and there were no differences in its incubation period (about 12 days), or its character or course, to indicate that acquired resistance to the Brown-Pearce tumor influenced it. But more extensive and delicate tests are called for.

## GENERAL DISCUSSION

Some of the characters of the papilloma have been compared with those of the neoplasms as they were described. It remains to survey the findings in their general relation to the tumor problem.

The Shope papilloma, as occurring in nature, manifestly falls into the group of the infectious warts, condylomas, and papillomas, pathological processes of such dubious character that they have no fixed habitation in textbooks, some authorities classing them with the tumors and others with the hypertrophies or the inflammations. recent reviews deal with those known to be due to viruses (16). have been encountered in several animal species. In 1920 Magalhaes stated in a preliminary report (17) that he had produced generalized papillomatosis in steers by the intravenous injections of filtrates and suspensions of tissue from a spontaneous instance of the disease. No detailed account of the work has appeared. Ullmann (18) demonstrated the presence of a virus in a laryngeal papilloma of a boy, by two successive transfers to the skin of adult volunteers, the second of which gave rise to a papillomatous growth which developed so

The rabbit papilloma differs in several important respects from those previously studied experimentally. No development of secondary rowths takes place by infection from an existing papilloma, a frequent appening in human beings with warts or condylomas, and in dogs arrying the pointed condyloma of Borst (19) or the infectious papilma described by DeMonbreun and Goodpasture. Owth does not show any morphological peculiarities that enable one

to distinguish it from the tumors. Unlike the generality of infectious warts, papillomas, and condylomas it does not tend to retrogress and disappear, but usually, in experimental instances at least, continues its proliferation indefinitely. Furthermore the virus is capable of causing vigorous, progressive growths in animals of an alien genus (domestic rabbits).

## Tumor Attributes of the Growth

Whatever one calls or however one classes the papilloma it possesses, as our experiments have shown, the traits of behavior and appearance which characterize the tumors. It is an autonomous new growth, purposeless, parasitic, and, on occasion, progressive. Its extension on the skin ceases after a time because local conditions become unfavorable to it; but it continues to proliferate there and can be stimulated to renewed aggression. When implanted within the host it often looks and acts like a highly malignant neoplasm, directly invading and destroying the normal tissues, and causing death. On excision it recurs unless removed entirely; and during operation its cells may undergo implantation in the tissue of the wound or upon the peritoneal surface, with result in secondary nodules. Its own proliferation invariably involves it in widespread necrosis, and it may retrogress at some situations while progressing at others. Scharlach R stimulates it and bacterial infection sometimes causes it to become pronouncedly malignant. Its morphology as well as its behavior are influenced by the peculiarities of the individual host. It frequently penetrates into the blood and lymph vessels; but though the injection of fragments of it into the circulation not infrequently results in lung nodules. owing to cell survival and proliferation, conclusive instances of metastasis formation as distinguished from accidental implantation have not thus far been encountered.

<sup>&</sup>lt;sup>4</sup> The occasional retrogression in cottontails of the experimentally induced growth, and the occurrence of individuals resistant to the virus and possessing neutralizing antiviral bodies in the blood, suggest that under natural conditions retrogression may sometimes occur, or else that the papilloma may be torn off the skin, or gnawed away completely, after the hosts have developed neutralizing antibodies for the virus as such.

The papilloma produces no distinctive cytological alterations in the blood or in the other organs. It elicits a stroma and usually some new formation of connective tissue attributable to bacterial contaminants, but no characteristic histological reaction occurs about it inflammatory changes found at its base, when long established on the skin, are referable to trauma, necrosis, and incidental infection.

The morphology of the growth when proliferating within the body under favorable circumstances is that of an epidermoid carcinoma. Its cells no more give indication of the presence of an extraneous cause than do those of such tumors. When situated on the skin it closely resembles them in its histological relations to the surrounding epidermis. Virus inoculations may cause it to develop at one point or many, according to the technique employed; but the established growths appear to enlarge solely by a multiplication of their own cells, and the experimental evidence provides reasons for supposing this to be the case. In some animals the experimentally induced papilloma retrogresses, and when so doing it goes through the identical histological changes recorded for the epidermoid carcinomas of man and of rabbits, with a similar cellular reaction in the surrounding tissue. On transfer to other hosts the fate of the papillomatous tissue appears to be wholly uninfluenced by the association with it of a causative virus to which as such the host may be susceptible, but is determined instead by the laws governing the transplantation of tissues generally, including tumor tissue. As in the case of the chicken tumors, two sorts of resistance can be detected in animals to which the growth is transferred, these being directed against the strange cells and the agent responsible for their pathological behavior, respectively.

The habit of the tar tumors to become malignant or retrogress in response to local stimulating or repressing influences has been many times invoked as proving that neoplasms in general are the consequence of intrinsic cellular modifications. The papilloma, a growth rabbits frequently cease to enlarge after a time, like the papilloma when it is situated on the skin. In both cases a dense layer of newformed connective tissue is to be found next the growth, and in both here is evidence that it has checked the latter (20).

Comparison with the Chicken Tumors Andrewes (21) has recently assembled and discussed a graded series Andrewes (21) has recently assembled and discussed a graded series of disease processes due to viruses, ranging from such as are necrotizing to those causing growths of neoplastic character, namely the mg to those causing growins of neophastic character, namely the chicken tumors. The Shope papilloma belongs at the further end of the chicken tumors. the series; and some comparison of it with the chicken tumors would seem called for, though of necessity this must be confined to major differences and likenesses. The growths of both sorts resemble the uncrences and menesses. The growing of both sorts resemble the recognized tumors in their manifestations within the individual host, even as regards characters demonstrable only by experiment, yet both are due to filterable agents which can readily be separated from the are one to microunce agence which can readily be separated from the tissue. Each causative agent is specific for cells of a certain kind, affecting no others and making these behave in ways from which there is remarkably little deviation, the result being the regular production of an osteochondrosarcoma, for example, or a fissured sarcoma of intracanalicular pattern in fowls, or a papillomatous epithelial growth in rabbits. Nevertheless some of the agents will act upon the cells of an alien species (duck (22), pheasant (23) in the case of certain chicken anen species (auca (22), pheasant (20) in the Case of certain chicken tumors, domestic rabbits in that of the Shope papilloma). Once the cells have been acted upon, however, their character and activities are responsible for the obvious disease phenomena. Cell injury is requisite responsible for the obvious ansease phenomena. Centulary is requisite to the action of the agent causing Chicken Tumor I (24), as also to that of the virus producing the rabbit papilloma.

Theoretical difficulties in supposing tumors to be due to viruses, and certain characters of the agents causing the growths in fowls, have led some workers to assume that these agents are substances elaborated by the cells themselves; but experiments in this direction have but enlarged the similarities to the viruses, as Andrewes has pointed out. The agent causing the rabbit papilloma has the typical charout. The agent causing the fault papinoma has the typical charge acters of a virus, and the disease to which it gives rise is endemic in the wild rabbits of certain localities, multiple growths being not infrequent. The incidence of the chicken tumors on the other hand does not suggest an infectious source. But the conditions favor transfer of the papilloma virus. It persists in active form in the dried tissue covering the growth (a fact demonstrated incidentally in Experiment 3 of our transplantation series), and some of this must often b

rubbed off into the dust of the rabbit's burrow or form. It is also notably resistant to heat (25),—which it must frequently have to withstand on the prairies where the disease is found. Acting as it does upon freshly injured epidermis, every rabbit with a scratch provides an opportunity for it, while furthermore the great majority are susceptible. The activity of the agents present in chicken tumors, those thus far studied at least, is easily destroyed, and their distribution to the outside world is beset with difficulties. The growths lie in the interior of the body, and the causative agents, if entering or leaving cells, must in many cases have to run the gauntlet of neutralizing substances present in the blood. Yet if they are to produce the disease in other fowls they must somehow reach injured cells that are beneath the body surface. Such differences as these in the conditions determining effectiveness may account for a difference in incidence that at first glance seems fundamental.

# Virus Changes and the Behavior of the Growth

There is good reason to believe that once the agents causing the rabbit papilloma and chicken tumors have become associated with the cells they, like other viruses (26) and certain intracellular bacteria (27), are protected from the influence of neutralizing principles in the body fluids. Growths of noteworthy vigor are to be found in individuals completely resistant on inoculation with the causative agent as such and possessing antibodies effective against it in their blood. The behavior of both the papilloma and the chicken tumors is directly referable to that of the cells composing the growths. Yet alterations in the causative entities cannot but influence the latter profoundly. Attenuation of the agent responsible for Chicken Tumor I, by heating, prolonged desiccation, or a sojourn in glycerin, leads it to produce tumors that progress slowly or retrogress; and heating the virus of the papilloma at 67°C. for 30 minutes (28) results in retrogressing growths. The agent responsible for Chicken Tumor I cannot be re-

In a recent, unpublished study of the age incidence of neutralizing substances for the agent causing Chicken Tumor I (McMaster, Hudack, and Rous), it was found that the majority of normal, adult Plymouth Rock fowls possess them, whereas they are much less frequent in pullets of the same stock, and in the pooled serum of new-hatched chicks they are not demonstrable.

covered from slowly enlarging sarcomas (29), and the papilloma virus, though setting up a vigorous and persistent proliferation in domestic rabbits, undergoes some change in these that robs it of uomesuc randres, undergoes some change in the modified in yet infectivity even for wild rabbits. That it may be modified in yet another way is suggested by the tendency of implantation growths anomer way is suggested by the century of implantation growins in some animals, domestic ones especially, to enlarge more slowly as in some animals, domestic ones especially, secondary retrogression is time passes and eventually to retrogress. frequent in the case of some of the chicken tumors also. In each case the cell or its immediate milieu is the medium in which multiplication takes place of the entity causing the disease. Presumably it becomes attenuated when this medium is not wholly suitable and in conse quence the cell proliferation it induces becomes slower or stops.

The papilloma differs significantly from the tumors in the conditions of its natural occurrence. Its incidence shows it to be an infectious disease, and its virus cause need only be brought in contact with traumatized epidermis for a growth to result. Few facts have been so elaborately proven for the tumors as that their incidence is without sign of an infectious cause; while furthermore all efforts to demonstrate sign of an infectious cause, while failed. Rabbits can be protected from such a cause have consistently failed. the papilloma: when kept where there is no virus their skins can be repeatedly scarified without inducing it. But the most careful isolation fails to give the least protection from cancer. Mice which are tarred while living on sterilized bedding in sterile jars, drinking sterile water, and eating muscle removed under aseptic conditions from other water, and caung muscle removed under asoptic continuous from other species, develop tumors with the same frequency as do controls in dirty surroundings, exposed to every chance infection, and fed on a highly

Another difference from the tumors is to be found in the local conditions leading to occurrence of the growths. Both arise in tissue that has been rendered abnormal in some way. But the papilloma various diet (30). will develop on the basis of acute injury, a scratch sufficing to render the epithelium susceptible to the virus, whereas the generality of tumors follow only upon long-continued disturbance (31), the precise character of the cellular changes preliminary to the neoplastic process being not yet clear. The papilloma appears a few days after injury

attended by introduction of the virus; and about the beginning growth there is little or no cellular reaction. The typical epidermoid tumors, on the other hand, of rabbits, mice, and men, develop only after months or years respectively, of recurring local disturbance; and where they appear marked "precancerous changes" are ordinarily present in the tissue.

# Implications for the Tumor Problem

In thinking upon the tumor problem facts deserve recognition before new biological possibilities are invoked. One of these facts is that an epithelial growth due to a filterable virus, namely the rabbit papilloma, as observed in the individual host, acts and looks like a tumor. Can one suppose that tumors in general are due to viruses or other extraneous entities? The supposition is tenable only if such entities are widely distributed throughout the animal population, being constantly present in or upon the body, like the colon bacillus or the staphylococcus; and if their opportunity to cause tumors is restricted by the need for very special conditions. Ubiquitous such entities would have to be to produce cancer in human beings everywhere throughout the world, and with the same frequency in isolated, tarred mice as in unsheltered ones. If they acted under ordinary conditions multiple spontaneous growths would be frequent, and the general incidence of tumors would provide evidence of an infective cause for them. Each of the two requisites for the assumption that extraneous entities cause tumors is manifestly a corollary of the other. considerably an agent is conditioned in its activity, the more often must it be present if it is to cause disease at all. Though widely present, if heavily conditioned it will cause disease only here and there and now and again. A presumptive instance of how the combined influence of several factors modifying the effectiveness of a causative entity may determine the incidence of the neoplasms has already been discussed during the comparison of the chicken tumors with the papilloma. Evidence that the mammalian tumors are heavily conditioned in their occurrence is at hand, both in the history of indi-

<sup>&</sup>lt;sup>6</sup> Andrewes has recently advanced the view that tumors are due to viruses and as discussed comprehensively the facts for and against this conception. (An-

vidual cases and in the data on "precancerous changes." Yet though this holds for most instances, where the local circumstances are exceptionally propitious numerous spontaneous tumors may arise, as where a broad expanse of skin has been damaged by the Roentgen ray, by tar, or other physical or chemical agency; and where the necessary general conditions are fulfilled, as in certain races or families, case incidence may be high. The demonstration of the cause for the generality of tumors, whatever this is, waits upon the provision by the investigator of the conditions necessary to its effectiveness.

Experimental study of the rabbit papilloma of Shope, a growth caused by a virus, has shown that it possesses the immediate characters whereby tumors are recognized. Often it looks and acts like a malignant neoplasm. It differs from the tumors as a group, however, a manguant neopiasm. It omers from the tumors as a group, nowever, in its incidence which is that of an infectious process, and from other mammalian tumors in that its cause has been demonstrated. The possible bearing of the findings upon the problem of tumor causation is discussed. The morphology and behavior of the generality of tumors can no longer be taken to exclude the possibility that these are produced by extraneous, living entities. The incidence of some of the tumors at least, and the failure to demonstrate their cause can both be explained on the assumption that they are due to such entities, widely distributed in or upon the animal population but effective only under special circumstances. Present knowledge makes this assumption reasonable as a basis for further work.

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## EXPLANATION OF PLATES

## PLATE 45

Fig. 40. Cells in the granular layer of epithelium at the bottom of a crypt between two papillae,—from a papilloma on the skin of a wild rabbit (W.R. 19)

PEYTON ROUS AND J. W. BEARD killed 60 days after inoculation. In the cytoplasm are numerous moderately refractile bodies, which appear to have developed from the granules characteristic

the underentiating centrajer. A 023.

The Fig. 41. Unusually marked cellular reaction about beginning growths. The specimen was excised 12 days after virus fluid had been rubbed into the scarified of the differentiating cell layer. X 625. specimen was excised 12 days and virus mad been rubbed into the scarmed skin of the abdomen of a domestic rabbit. The four little growths here shown are extending down into the connective tissue, and keratinized epithelium has begun to heap up over them. About each is an accumulation of round cells, with an The marked capillary dilatation is occasional polymorphonuclear leukocyte.

Fig. 42. Graft from the leg muscle of a domestic rabbit dying of intercurrent causes 7 days after transplantation to it of fragments of papilloma from another domestic rabbit. The proliferating graft has rounded out and some extension from it into the surrounding tissue has occurred. A capsule is forming in which barely visible. Noming the surrounding assure has occurred. In capsure is forming in which lymphocytes are present. The dark strip is epithelium overstained with methy-

lene blue. × 65.

Fig. 43. Reaction about papilloma fragments transplanted from a domestic ric. 45. Reaction about papinoma fragments transplanted from a domestic All of the implanted rabbit into the leg muscle of a wild one 37 days previously. rappir into the leg muscle of a wird one of the size and shape of the largest fragment tissue is now dead and fails to stain; but the size and shape of the largest fragment ussue is now used and lans to stam, but the size and shape of the largest tragment shows that it had undergone some proliferation. Lymphocytes have accumulated in enormous numbers and encapsulation has taken place.

enormous numbers and encapsulation has taken place. X 40.

Fig. 44. Edge of a retrogressed nodule situated in an old laparotomy wound (Domestic Rabbit 1-21). The dead squamous epithelium is surrounded by granulation tissue containing giant cells. X 100.

Fig. 45. To show the influence of Scharlach R on the development of the papilrig. 45. 10 show the inhuence of Scharlach R on the development of the papilloma. Virus was tattooed into five spots in a line across the middle of the ear ioma. Virus was tattooeu into live spots in a line across the initiadermal injection (A), and also into three spots near the tip, 5 days after an intradermal injection (A), and also into the middle one (M) of the three. Four similar tattooings were also or the aye into the muone one (111) or the times. Four summar factoomies were also done into the skin near the base, which had been widely infiltrated with Scharlach none into the skill hear the base, which had been widely minitrated with scharizer R at the same time as the tip.

The photograph was taken 26 days later when the K at the same time as the up. The photograph was taken 20 days later when the growths in the normal skin were still small. Wherever the virus was introduced growins in the normal Skin were sum sman. Wherever the Virus was introduced into tissue affected by the dye relatively large growths have appeared, one near the into tissue affected by the dye relatively large growths. base of the ear developing as a spherical nodule (S) under intact skin. Still pase of the ear developing as a spherical nodule (c) under meact sain. Sun nearer the base, where there had been no inoculation, two smaller nodules have nearer the base, where there had been no moculation, two smaller notines have also appeared, one papillomatous in character (P), the other an epithelial cyst also appeared, one paphomatous in character (r), the other an epithelian cyst.

(C) referable merely to the Scharlach R. The character of the growths was

termined by biopsies. A 72. Fig. 46. The localization of circulating papilloma virus. Scharlach R was injected intradermally into fourteen spots in a circle on the side of a domestic pected intradermany into sourceen spots in a circle on the side of a domestic rabbit, and 18 days later the injection was repeated. Well defined, discoid determined by biopsies. X 1/2. ranner, and to days later the injection was repeated. Wen defined, discoid thickenings of the skin resulted, with marked scurfiness.

injection, 5 cc. of virus fluid was injected into the blood. After another 10 days seven of the discs were noted to be everywhere thickened and raised, whereas the others were disappearing; and soon the papillomatous change had declared itself.

Fig. 47. Unusually sharp demarcation between the normal epidermis and that affected by the virus. Note the pathological enlargement of the cells and nuclei, the crowding, and the darker staining with methylene blue. The section was taken from the edge of an enlarging, pigmented papilloma 22 days after it had appeared on the skin of a domestic rabbit subjected to punctate inoculation.

Fig. 48. Edge of a growing skin papilloma isolated from the surrounding epidermis by a tear. Specimen taken from a domestic rabbit inoculated 48 days previously.

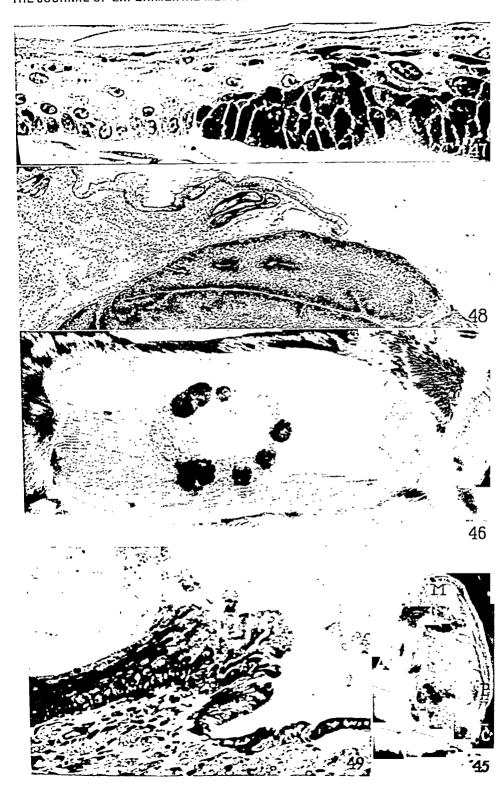
Fig. 49. Junction of papillomatous epithelium and that of the kidney pelvis. The animal (Wild Rabbit 10) had a narrow horn of keratinized epithelium projecting into the pelvis, as result of an implantation made 79 days previously through a hollow needle (see Paper I). The section is from the edge of the base of the horn.



Photographed by Louis Schmidt

(Rous and Beard: Virus-induced mammalian growth III)





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### ULTRAFILTRATION OF THE VIRUS OF POLIOMYELITIS

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That the inciting agent of human poliomyelitis should be classified with the filtrable viruses has been recognized since the pioneer work of Landsteiner and Levaditi (1) and Flexner and Lewis (2), who showed that this virus passes through Berkefeld filters. Efforts to determine more nearly the size of the virus were made by Amoss (3), who showed that it passes easily through a Berkefeld V filter, less easily through an N filter, and is diminished in quantity after passage through a W filter. More accurate methods for determining the size of virus particles have been made available by the important work of Elford, who has described a method for preparing collodion membranes of graded porosities, and has shown that it is reliable (4). Using his methods, we have obtained the additional evidence here presented regarding the size of the poliomyelitis virus particle.

### EXPERIMENTAL

The graded collodion membranes used in these experiments were prepared according to the method of Elford, with certain minor modifications. The technic of preparing the membranes and the apparatus used have been described by Bauer and Hughes (5).

Six filtration experiments were carried out. In each experiment five to eight membranes were used. These varied in porosity from 30 m $\mu$  to 265 m $\mu$  average pore diameter, and in thickness from 0.12 mm. to 0.22 mm. The filtration area was approximately 5 sq. cm.

The strain of poliomyelitis virus used throughout these experiments was the M.V., for which we are indebted to Dr. Simon Flexner. This strain was chosen on account of its high and constant virulence.

Preparation of Virus Suspensions.—Infective spinal cords to be used for the preparation of virus suspension for filtration purposes were removed from monkeys

shortly after the onset of paralysis. The incubation period of these monkeys, i.e. the number of days elapsing between inoculation and onset of paralysis, varied from 7 to 11 days. On account of the low concentration of virus in the brain, only the cord was used. In the first four experiments the whole cord was used, but in the last two only the cervical and lumbar enlargements were used. infective tissue was ground thoroughly in a mortar with quartz sand and a suitable The concentration of tissue in these suspensions varied from 4.1 to 10 per cent by weight. The virus suspensions were then centrifuged for from 30 to 90 minutes at 3,000 R.P.M. In the last four experiments an angle centrifuge was used. The supernatant fluid was then passed through a Seitz filter. was distinctly turbid because of the presence of finely divided fat. It is essential that the material to be filtered through the finer collodion membranes should be absolutely clear. Consequently, attempts were made to clarify the Seitz filtrate further by passing it through coarser membranes prior to filtration through membranes of fine porosity. It was found, however, that the fat globules passed through membranes with an average pore diameter of 500 m $\mu$  or greater, and the filtrate of those membranes had practically the same turbidity as the original Seitz filtrate. On the other hand, when membranes with an average pore size of 300 mµ were used, the filtrates were clear but the membranes became clogged completely after the passage of a few cubic centimeters. Attempts were also made to use the sand and paper pulp filters described by Galloway and Elford (6) for the clarification of the monkey cord emulsion, but these attempts were unsuccessful in that the filters invariably became clogged after the passage of a few cubic centimeters of the suspension. It was therefore decided to use straight Seitz filtrates as stock filtrates for passage through the finer graded membranes for the determination of the filtration end-point of the virus.

The composition of the diluent used for the suspension of finely ground monkey cord varied in each experiment, as shown in Table I. As indicated in this table, hormone broth and phosphate buffer solutions in varied proportions were incorporated into the diluent used in all experiments. The importance of the broth in ultrafiltration has been pointed out by Galloway and Elford (6) and Bauer and Hughes (7). In one experiment 5 cc. of broth were passed through the membranes prior to the filtration of the virus suspension.

All filtrations were carried out under a positive pressure of nitrogen. amount of pressure applied was 100 cm. of mercury, except in one experiment in which three atmospheres were employed. The amount of filtrate collected varied from 5 to 10 cc. The protein content of the filtrates was determined in every instance. The infectivity of the filtrates was tested by the intracerebral injection of monkeys. The amount inoculated was 1 cc. in each experiment, except in Experiment 4 when 2 cc. were injected. Monkeys were kept under observation for at least 5 weeks. Diagnosis of poliomyelitis was made on clinical grounds; but most of the monkeys inoculated with the filtrates were killed after the development of paralysis, and the diagnosis was confirmed by histopathological

TABLE I

# Details of Methods Followed in Filtration of Poliomyelitis Virus

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
Source of virus	1 whole cord	2 whole cords	1 whole cord	1 whole cord	Cervical and lumbar callargements of 3 cords	Cervical and lumbar enlargements of 5 cords
Percentage of in- fective tissue in stock suspension	4.1	10.0	5.0	5.0	5.0	8.9
Composition of diluent	Hormone broth pH 8.0, 25 cc. Phosphate buffer solution x/15, pH 8.0, 50 cc. Distilled water 20 cc. Normal monkey serum 5 cc.	Hormone broth pH 8.0, 50 cc. Phosphate buffer solution x/15, pH 8.4, 25 cc. Distilled water 25 cc.	Hormone broth pH 8.0, 25 cc. Phosphate buffer pH 8.4, 25 cc. Ascitic fluid 25 cc. Distilled water 25 cc.	Hormone broth pH 8.0, 50 cc. Phosphate buffer at/15, pH 8.0, 50 cc. Normal monkey serum 5 cc.	Hormone broth pH 8.0, 50 cc. Phosphate buffer pH 8.0, 50 cc. Normal monkey serum 5 cc.	Hormone broth pH 8.0, 40 cc. Phosphate buffer xt/15, pH 8.2, 40 cc. Normal monkey serum 5 cc.
Filtration pressure	100 cm. Hg	228 cm. Hg	100 cm. Hg	100 cm. Hg	100 cm. Hg	100 cm. Hg
Amount of filtrate injected into mon- keys	1 cc.	1 cc.	1 cc.	2 сс.	1 cc.	1 cc.
Amount of filtrate collected	6-10 сс.	6-10 cc.	8 cc.	5 cc.	5-6 cc.	5.5-6.5 cc.

study. For controls one or more monkeys were injected with the stock Seitz filtrate in every instance. In several experiments the infectivity of the Seitz filtrate dilution 1/10 and 1/100 was also tested. The immunity of the monkeys that remained well after injection with filtrates was tested in later experiments

TABLE II Results of Filtration Experiments with Poliomyelitis Virus

	Membrane			1	" Foliomy	clitis Virus
No.	Average pore diameter	Thickness	No. of experi- ments	No. of monkeys inoculated with filtrates	No. of monkeys developing poliomyelitis	Incubation period
itz filtrate 1, tz filtrate 1,	215 100 100 90 67 67 65 65	19   15   2   4   3   3   1   3   1   6   6   3   4	17	1 1 1 2 1 1 1 2 2 1 2 2 1 2 4 6 1 6 1 6 1 0 0 0	1 1 1 2 1 0 0 0 0 1 8 1 1 0 0 0 1 1 0 0 1 0 0 1 2 31 0 0 1 2 7 8 1 1 2 7 8 1 1 8 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	days 17 16 14 10, 19 3

by intracerebral inoculation of stock Seitz filtrates. additional control animals for the infectivity of the stock virus filtrate. These monkeys served as

Results of Experiments.—The results of the filtrations are shown in Table II. The irregularity of the results obtained is probably due to

the well known wide range in susceptibility of *rhesus* monkeys to the virus of poliomyelitis. This is accentuated when filtrates are used which certainly have a much smaller concentration of virus than is present in the suspensions of virus commonly used in work with poliomyelitis.

On two occasions the virus was demonstrated in the filtrates of the membranes with an average pore diameter of 40 m $\mu$ , and on one occasion in the filtrate of a 35 m $\mu$  membrane. No monkeys became infected with the filtrates obtained from a membrane of 30 m $\mu$  average pore diameter. The filtration end-point of the virus of poliomyelitis in our experiments is consequently 35 m $\mu$ . In view of the fact that in filtration experiments a considerable amount of protein is adsorbed in the membranes, which necessarily reduces the diameter of the pore, Elford (8) has estimated that with membranes with an average pore diameter greater than 10 m $\mu$ , a particle must have a diameter not greater than from one-third to one-half of that of the pore in order to traverse it. Applying this formula to our results, it would seem that the particle size of the poliomyelitis virus lies somewhere between 12 and 17 m $\mu$ .

### DISCUSSION

Our results show that the virus of poliomyelitis is extremely small, approximating that of foot-and-mouth disease (8-12 mu, Galloway and Elford (6)), and it would seem probable that it is even smaller than our results would indicate. In evaluating ultrafiltration experiments, all that can legitimately be concluded is that under the conditions of the experiments the virus passed through membranes of a certain pore size. The observations are of only minor value unless the filtrations are carried out under optimum conditions. Such conditions were by no means present in our experiments. Minute particles of fat, which passed through the Seitz filter and through the coarser membranes, interfered considerably in the filtration through membranes of smaller porosities. Furthermore, Galloway and Elford (6) have shown that in ultrafiltration experiments the concentration of virus is of great importance and that only a high concentration of virus in the stock filtrate gives a sharp filtration end-point. Poliomyelitis virus is present in infective cords in relatively small amounts.

In our experiments on three occasions the infectivity of a 1/100 dilution of the Seitz filtrate was tested by the intracerebral injection into monkeys. Only once was virus demonstrated. Another factor of importance which renders the interpretation of the results difficult is the wide variation in the susceptibility of the monkeys to minute doses of the virus.

### CONCLUSIONS

From the ultrafiltration analysis the size of the virus of human poliomyelitis has been estimated to be somewhere between 12 and 17 m $\mu$ . Technical difficulties encountered and the low concentration of the virus make it seem possible that the virus is even smaller.

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# THE PROBLEM OF THE SIGNIFICANCE OF THE INCLUSION BODIES FOUND IN THE SALIVARY GLANDS OF INFANTS, AND THE OCCURRENCE OF INCLUSION BODIES IN THE SUBMAXILLARY GLANDS OF HAMSTERS, WHITE MICE, AND WILD RATS (PEIPING)

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Plates 47 and 48

(Received for publication, September 24, 1934)

In recent years inclusion bodies have been observed with increasing frequency both in man and in animals. Although in many instances these pathological changes have been associated with definite virus diseases, for example poliomyelitis (1, 2), Rift Valley fever (3), yellow fever (4, 5), infectious ectromelia (6), etc., they have also been frequently reported as accidental findings.

The constant association of inclusion bodies with well established viruses such as those of variola, vaccinia, herpes, sheep pox, fowl pox, Virus III, the submaxillary gland virus of guinea pigs, etc., has led many workers to assume as a working hypothesis that the finding of these cytoplasmic or intranuclear changes means the presence of a filtrable virus. Thus recently Feyrter (7), McCordock (8), Rich (9), and McCordock and Smith (10) have found inclusion bodies in the lungs of children dying of postpertussis pneumonia, and have therefore suggested that whooping cough may be due to the action of a virus, or to the combined action of a virus and the Bordet-Gengou bacillus.

Cowdry (11) takes exception to this point of view. It is, of course, entirely possible, as he states, that eventually we shall be able to produce inclusion bodies by agents other than filtrable viruses, but in the meantime it seems logical to continue searching for a virus whenever these characteristic lesions are found. At the present time our knowledge of filtrable viruses of low pathogenicity is practically nil,

Inclusion Bodies Reported	Free J.
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Inclusion	-

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,	thoms of Disease	Author	1 4	by Farber and Wolbach (12)	McCordock and Smitt	(10)	Russell (15)		~	_ , , ,	Stewart and Rhoads (16)	Covell (17)		Hindle and Stevenson (18)	
	Specific Sym	Attempts at transmission	None	3	Negative	Nono	2001	<b>3</b> 3				 			3
TABLE I	ies No. ex- No. Per cent amined positive positive Age Age Disease	5 still-birthe	45 infants Salivary glands, etc.	Infants Lung, salivary	Stand, liver, etc.	Brain: spongioblastoma multi.	Brain: miscellaneons with	Liver: Hodgkin's lymphogranu-	Liver: death due to miscella-	Nasal mucosa		Lung, nasal mucosa, trachea, bile duct, bronchiole	Kidney tubule	מ	
Reporte	o. Fer cen	<u> </u>			- 19	5 =	36	10		·!	33				
Bodies	ex- N ned posi	51		14	51	12	10	S	19		20				
lusion	No.					108	36	56	31		99				
Inc	ğ	Man	Man	ı	Man				Monkey (M. rhesus)	Monton		Monkey (M. rhesse)	Monkey (Cer-	- chuncens)	

Dog	89	2	3		Liver	Negative	Negative   Cowdry (19)
Dog					Brain; nerve and glia cells	2	Dawson (20)
Rat (wild)			8		Kidney tubule	None	Hindle and Stevenson (18)
Rat (white)	20	10	#	2 mos.	Ducts of submaxillary gland	*	Thompson (21)
Mouse (Clacton strain)	25	25	100		Liver	Positive	Findlay (14)
Guinca pig			22	Full grown	Ducts of submaxillary gland	*	Cole and Kuttner (22)
Rabbit			8	3	Testes	3	Rivers and Tillett (23)

and it is especially in this type of disease that inclusion bodies may prove of aid. The finding of inclusion bodies in various internal organs of still-births (12) is difficult to attribute to an infectious agent, but it may be that we are dealing with a filtrable virus of low virulence, and that infection has taken place in utero. Wagner (13) states that the occurrence of these peculiar structures in still-born and premature infants is against their being of protozoan origin, since the human placenta is impermeable to parasites of this size. A filtrable virus, on the other hand, could pass the placental barrier. Whether this agent, if it is a filtrable virus, is frequently present in the circulation of adults, or has a greater tendency to invade the blood stream during pregnancy, and thus infect the fetus, remains a matter of speculation. The fact that these lesions occur in infants in the complete absence of symptoms, cannot be held to exclude the possibility of a virus, since Virus III and the submaxillary gland virus of guinea pigs were discovered accidentally in the course of experimental work, and did not produce symptoms. Findlay (14) has recently demonstrated a filtrable virus in the parenchymal cells of the liver in a certain strain of mice, which is also symptomless.

In spite of the fact that inclusion bodies have been observed as accidental findings in man and in many species of animals, relatively few attempts have been made to determine their significance. Table I summarizes the occurrence of inclusion bodies reported in the absence of specific symptoms, and the number of instances in which attempts at transmission have been made.

It will be seen that 6 of the 13 workers who have recorded these observations attempted to demonstrate the presence of a virus. 3 of the 6 attempts proved successful.

In analyzing the cases of infants previously reported, it is of interest that in those instances in which the submaxillary and parotid glands are reported as involved, inclusion bodies are not found in other tissues of the body. It is also worthy of note that in still-births and in the very youngest infants, the submaxillary and parotid glands are often negative, and the kidneys, lungs, and liver most frequently show the hypertrophied cells containing intranuclear inclusion bodies. This suggests that if these changes are due to a filtrable virus and if the infection has taken place in utero, as must be assumed, the virus first

attacks the viscera and then gradually localizes in the salivary glands. On the other hand, it may be that we are dealing with different viruses.

In view of the fact that the pathological findings in the submaxillary gland disease of guinea pigs are so similar to those found in the submaxillary and parotid glands of infants dying from miscellaneous causes, it seemed of interest to try to determine whether a virus could be demonstrated in autopsy material of this kind. Moreover, before any special importance can be attached to the finding of inclusion bodies in pathological conditions occurring in children, it would be important to try to discover the significance of intranuclear changes found in the salivary glands of young children in a fairly high percentage of routine autopsies (Farber and Wolbach, 12 per cent (12)). the time the present work was started, no experiments to demonstrate a virus from human material of this kind, had been made. In a recent publication McCordock and Smith (10) state that they have been unable to transmit a virus from the salivary glands of infants showing inclusion bodies, to young guinea pigs. Their experiments are not reported in detail.

# The Occurrence of Inclusion Bodies in the Submaxillary Glands of Chinese Still-Births and Infants Less than 2 Years of Age

Method.—The submaxillary glands, and whenever possible the parotid glands, were obtained from autopsies on still-births and infants less than 2 years old. The autopsies were performed anywhere from 12 hours to 4 days after death. No precautions for asepsis were observed in removing the glands, but they were carefully washed with sterile saline. A small piece of tissue was removed from each gland for section, and the remainder was placed in 50 per cent glycerine. After 3 to 5 days exposure to glycerine in the ice box, some of the material was washed, ground with sand, and centrifugalized at low speed. The supernatant fluid was then injected intracerebrally or directly into the submaxillary glands of a variety of animals, young guinea pigs, hamsters, mice, rats, rabbits, and monkeys. All intracerebral injections and removal of glands from animals were carried out under ether anesthesia. The rest of the tissue was stored in glycerine until the histological sections had been examined. In the earlier experiments all the material was inoculated into animals as soon as possible without waiting for the completion of the sections; in later experiments the tissue obtained from stillbirths was stored in glycerine until the sections had been examined, and if these were negative, no animal injections were made. Aerobic and anzerobic cultures of the supernatant fluid were made before inoculation. In spite of the exposure to glycerine some of the material was not sterile, and a small number of organisms of a part of each gland at the time of transfer. Although some of the glands showed a slight amount of cellular reaction, most of them were negative.

No evidence was obtained that the agent which had produced the characteristic changes in the submaxillary and parotid glands of this infant had been transmitted to rabbits, guinea pigs, or mice by intracerebral or intraglandular injection.

Case 2.—The submaxillary and parotid glands were stored in glycerine for 3 days, Feb. 26 to Mar. 1, 1934. A part of each gland was emulsified and the supernatant fluid injected directly into the submaxillary glands of 2 guinea pigs and 2 mice. The glands were removed after 2 weeks, and sections prepared. Both the mice and the guinea pigs failed to show any characteristic reaction.

Case 3.—The submaxillary and the parotid glands were stored in glycerine for 5 days, May 2 to 7, 1934. A part of each gland was emulsified, and 0.1 cc. of the supernatant fluid was injected intracerebrally into 3 young guinea pigs. 2 of the guinea pigs were on a normal diet and 1 was on the vitamin-deficient diet used by Zinsser et al. (24) in their work on typhus. Cultures made from the supernatant fluid showed a few colonies of B. coli. All 3 of the animals remained well and were discarded.

On June 6, 1934, it was found that the sections of the glands from this case showed many inclusion bodies (see Fig. 2). The material had, on this date, been stored in glycerine for a period of 34 days. The tissue was emulsified in the usual way, and the supernatant fluid was injected intracerebrally into one young (Macacus rhesus) monkey, approximately  $2\frac{1}{2}$  years of age, and intraglandularly into another, older monkey of the same species. Both monkeys remained well. The monkey that had been injected intracerebrally was killed on the 7th day. The brain was removed for histological examination. The inoculated gland was removed from the second monkey on the 15th day, and sections made.

Microscopic sections of the brain were negative. At the time that the submaxillary gland of the second monkey was injected, the other gland was removed for histological examination. Sections of the uninoculated gland showed very occasional small areas of lymphocytic infiltration. The inoculated gland showed a similar reaction which was somewhat more marked. The capsule of the inoculated gland was infiltrated with lymphocytes at one point. However, the contrast between the inoculated and uninoculated glands was not striking, and no inclusion bodies were found in either one.

No evidence was obtained that the salivary glands from this case contained a virus which could be transmitted to monkeys (*Macacus rhesus*).

Case 4.—The submaxillary and parotid glands were stored in glycerine for 18 days, June 18 to July 6, 1934. Attempts were made to reduce the resistance

of the animals to be inoculated, by exposure to X-ray, since Zinsser and Castaneda (25) have found this the most satisfactory method of reducing the resistance of rodents to typhus. Rabbits, guinea pigs, and rats were given the following dose immediately before inoculation: kv. 160, ma. 8, filter 5.0 cm. oil, 0.25 mm. Cu, 1.5 mm. Al, (effective wave length 0.19 Å. u.) distance 50 cm., 19 minutes, 400 Roentgen units. The tissue was emulsified in the usual way, and the supernatant fluid injected intracerebrally into 1 young rabbit, 2 young guinea pigs, and 3 young rats. All the animals remained well, and were discarded.

No evidence was obtained that rodents could be made susceptible to an infectious agent in the salivary glands by exposure to X-ray.

# The Occurrence of Inclusion Bodies in the Submaxillary Glands of Hamsters, White Mice, and Wild Rats

In the course of the attempts to transmit a presumptive filtrable virus from the salivary glands of infants to animals, the submaxillary glands of hamsters, wild and white mice, wild and white rats, squirrels, rabbits, and monkeys (Macacus rhesus), were examined histologically.

Hamsters.—It was found that the submaxillary glands of nearly all full grown hamsters showed characteristic pathological changes consisting of scattered areas of cellular infiltration composed mainly of mononuclear cells. In the vicinity of these areas, although usually not in them, one or two cells situated in the center of an acinus were greatly hypertrophied. The nucleus was also enlarged and contained an acidophilic inclusion body surrounded by a halo (see Fig. 3). staining of the inclusion body tended to be less dense at the periphery, but no definite structure could be made out. The inclusion bodies found in the acinal cells of the hamster are usually more regular in outline than those found in the guinea pig. There are often one or more small irregular basophilic masses lying close to the nuclear membrane. The halo is always well defined. The cytoplasm of the cell stains blue or purplish with eosin-methylene blue. In most instances the staining of the cytoplasm is irregular, some parts staining more deeply than others. Occasionally somewhat more definite basophilic masses could be made out in the cytoplasm. These changes suggest degenerative processes in the cytoplasm, rather than cytoplasmic inclusion bodies. The lesion differed from that found in the submaxillary glands of guinea pigs in that the acini and not the ducts, were primarily affected. In only three instances were these hypertrophied cells with acidophilic inclusion bodies, found in the duct as well as in the acini of the submaxillary glands of hamsters. Although the inclusion bodies found in full grown hamsters are smaller than those found in full grown guinea pigs, they can often be identified under the low power of the microscope. The fusion of two hypertrophied cells, each with its separate inclusion body, occurs fairly often in the

# ANGEOGRAPH BODIES IN SALIVARY GLANDS

The hamster virus survived for 6 days in 50 per cent glycerine. Longer periods of glycerinization were not tried. Attempts to transfer the virus from gland to gland were not undertaken.

# Intracerebral Injection of the Submaxillary Glands of Full Grown Mice into Young Mice Less than 1 Month Old

The submaxillary glands of full grown mice were examined histologically and it was found that a small proportion of them showed pathological changes similar to those observed in hamsters. About 20 to 25 per cent of the submaxillary glands of full grown mice obtained from the animal room stock of white mice (originally imported from the United States) showed scattered foci of mononuclear cells. In the vicinity of these areas, one or two cells, usually in the center of an acinus, were greatly hypertrophied, and the nucleus contained an acidophilic inclusion body surrounded by a halo (see Fig. 6). In mice these hypertrophied cells were never found in the ducts, only in the acinus. The lesion in mice was so similar to that in hamsters that it cannot be differentiated morphologically. Stock white mice in this laboratory were less frequently infected than hamsters, and the hypertrophied cells with the intranuclear inclusion bodies were never very numerous. In addition to the laboratory-bred white mice, the submaxillary glands of 6 wild brown mice were examined histologically. Although in four instances the glands showed foci of cellular infiltration, no hypertrophied cells with intranuclear

Attempts to demonstrate a virus in the submaxillary glands of mice which showed these lesions, were made. Young mice, less than 1 month old, usually failed to show any pathological changes and were

The submaxillary glands of full grown white mice were prepared in the same way as the hamster virus, and will subsequently be referred to as the "mouse virus." Although the submaxillary glands of 3 to 6 nice were combined, the sections made from a portion of each gland howed that they were never very heavily infected, and the characeristic cells were only found after considerable search. Intracerebral jections into young mice were difficult, and many of the animals ed as the result of inoculation. The successfully injected mice mained well for several days before they became sick. A few of em died on the 6th to 8th day. Sections prepared from the brains wed a slight localized meningeal exudate, consisting of mononuclear s. A few of these showed acidophilic intranuclear inclusion bodies.

# Localization of the Mouse Virus in the Submaxillary Gland Following Subcutaneous, Intraperitoneal, and Intraglandular Injection. Thermolability

The mouse virus was injected into a series of young mice, about 1 month old, from which one submaxillary gland had been removed for histological section—subcutaneously, intraperitoneally, and intraglandularly (submaxillary). At the same time mouse virus which had been heated at 60°C. for 30 minutes was similarly injected into another group of young mice. 2 weeks later the remaining submaxillary gland was removed for section from both groups of animals. Histological sections of all the mice which had received the unheated mouse virus showed varying degrees of cellular reaction and hypertrophied acinal cells with acidophilic intranuclear inclusion bodies, whereas the glands removed before injection were negative. The mice which had been injected with the heated material were also negative. These experiments indicated that exposure to 60°C. for 30 minutes destroyed the mouse virus.

No attempts to filter the mouse virus were made and its resistance to glycerine was not determined. Transfers from brain to brain or gland to gland were not undertaken.

A single attempt was made to transmit the mouse virus to guinea pigs which had been on a vitamin-deficient diet, but no characteristic lesion was produced.

# Experiments with the Rat Virus

Thompson (21) has reported the occurrence of intranuclear inclusion bodies in the duct cells of the submaxillary glands of 2 months old white rats. These lesions were present in 10 of the 70 rats examined. They were absent in 12 rats 6 months of age. Hindle and Stevenson (18) found inclusion bodies in the kidney tubules of wild rats caught in London. They do not state whether the submaxillary glands were also examined. Neither Thompson nor Hindle and Stevenson attempted to demonstrate a virus.

It seemed of interest to see if the stock white rats used in this laboratory showed similar lesions to those described by Thompson. Sections of the submaxillary glands of both full grown and young rats aged between 6 and 8 weeks have been entirely negative. On the other hand, about 50 per cent of the full grown wild rats caught in Peiping have shown lesions in the submaxillary glands, but not in the kidneys. The pathological changes in the submaxillary gland consisted of foci of mononuclear cells, and hypertrophied acinal or duct cells with acidophilic intranuclear inclusion bodies (see Figs. 7 and 8). In some of the wild rats only the acinal cells seemed to be involved, in others mainly the duct cells. The changes in the duct cells are similar to the lesions found in man and guinea pigs (see Fig. 9), and those in the acinal cells to the lesions found in hamsters and mice. A few experiments were undertaken to determine whether these pathological changes in the submaxillary glands of wild rats indicated the presence of a virus. Since young wild rats were not available, the submaxillary glands of wild rats were emulsified in the usual way, and injected intracerebrally and intraglandularly (submaxillary) into young white Two of the white rats injected intracerebrally became sick on the 8th day and were killed. Sections prepared from their brains showed a slight meningeal exudate consisting of mononuclear cells, a few of which contained acidophilic intranuclear inclusion bodies. The submaxillary glands which had been injected directly were removed after 2 weeks and showed a marked reaction consisting of a mononuclear infiltration with fairly numerous hypertrophied acinal and duct cells with acidophilic intranuclear inclusion bodies. maxillary gland removed before injection was entirely negative. These experiments indicate that the submaxillary glands of full grown wild Peiping rats sometimes harbor a virus which is transmissible to young white rats.

In the course of this work the submaxillary glands of 3 other animal species were examined: 3 normal full grown monkeys (Macacus rhesus), 3 large squirrels, and 5 rabbits. Scattered foci of mononuclear cells were found in all 3 of the monkeys, but no intranuclear inclusion bodies. The submaxillary glands of the 3 large squirrels were negative, but the submaxillary gland of 1 young squirrel showed an occasional focus of mononuclear cells, but no inclusion bodies. The submaxillary glands of the rabbits were negative, with the exception of 1 animal which showed a few mononuclear cells in one area.

## DISCUSSION

The occurrence of protozoa-like cells in the parotid glands of infants was first described by Ribbert (26) in 1904. Since that time, these structures have been frequently observed both in Europe and America. In examining the salivary glands obtained from routine autopsies on 24 Chinese infants, dying of various causes (exclusive of still-births), this lesion was found 4 times. This is as far as we know the first time that this lesion has been observed in Chinese children. Although our series is too small to draw any conclusions, it suggests that these pathological changes are fairly common in China.

In their discussion of the significance of intranuclear inclusion bodies frequently associated with whooping cough, McCordock and Smith (10) consider the possibility that the lesion found in the salivary glands of infants may be due to a virus closely related to the submaxillary gland virus of guinea pigs. Inoculation of human material into guinea pigs was, however, unsuccessful in their hands. In our attempts to demonstrate an infectious agent in the salivary glands obtained from 4 Chinese infants with characteristic pathological findings, guinea pigs, rabbits, hamsters, white mice, white rats, and 2 monkeys (Macacus Thesus) were inoculated intracerebrally or directly into the submaxillary gland. The results were entirely negative. Repeated transfers in rabbits, guinea pigs, and mice in the hope of adapting the "human virus" to a new host, were without avail. Efforts to reduce the resistance of the rodents by diet and X-ray failed. It is of course possible that the virus deteriorated quickly after death (the autopsies were performed 1 to 4 days after death), or that it is easily destroyed by glycerine. The material inoculated into monkeys had been stored in glycerine for 34 days.

In the course of these experiments, the submaxillary glands of several different species of animals were examined. It was found that the submaxillary glands of hamsters, white mice, and wild rats showed pathological lesions similar to those found in the submaxillary glands of man and the guinea pig. In contrast to the guinea pig, the lesion in hamsters and white mice usually involved the acinal rather than the duct cells. In wild rats (Peiping) both the acinal and duct cells showed the characteristic changes.

Emulsions of the submaxillary glands of full grown hamsters, white mice, and wild rats which showed these lesions, when injected intracerebrally into a young susceptible animal of the same species, often produced symptoms of meningeal irritation and death. Histological examination of the brain showed a localized, meningeal exudate consisting of mononuclear cells, some of which contained acidophilic intranuclear inclusion bodies. The lesion produced was analogous to that obtained with the submaxillary gland virus of guinea pigs. It was not possible to transmit the hamster virus or the mouse virus serially by intracerebral injection. It is an interesting fact that although intracerebral injections of the inclusion-containing submaxillary glands of rodents give rise to a fatal meningitis, the potency of the virus is decreased rather than increased, and it is only in the submaxillary gland itself that the virus can persist.

It was thought that if the hamster virus could be adapted to other rodents, it might become more virulent. The injection of the hamster virus by various routes, into rabbits, guinea pigs, and white mice, was without success. The intracerebral injection of the submaxillary gland virus of guinea pigs into hamsters was also negative.

The localization of the hamster virus and the mouse virus in the submaxillary glands follows subcutaneous, intraperitoneal, and intraglandular (submaxillary) injection in the respective species. The hamster virus is destroyed at 56°C. for 30 minutes, and is preserved in glycerine for at least 6 days. Longer periods of exposure to glycerine were not tried. The mouse virus is destroyed at 60°C. for 30 minutes. The thermolability of the rat virus was not determined.

The occurrence of inclusion bodies in the submaxillary glands of so many different species of animals, man, guinea pigs, hamsters, white mice, wild rats, is of interest. Although no inclusion bodies were found in the submaxillary glands obtained from 3 normal monkeys (Macacus rhesus) examined, areas of lymphocytic infiltration were present in all of them. In the various rodents studied, the submaxillary glands of very young animals were usually not infected, whereas in the human species typical lesions have been found in the viscera of still-births and infants that lived less than 48 hours. The age of the youngest child in whom inclusion bodies have been observed in the salivary glands is 2 months. The submaxillary gland viruses of

rodents are extremely specific, and it has been impossible to transfer the hamster virus to rabbits, guinea pigs, or mice, or the submaxillary gland virus of guinea pigs to hamsters. It is possible that the failure to transfer an infectious agent from the submaxillary glands of infants to animals is due to a very limited specificity and it may be necessary to inoculate higher apes before a positive result can be obtained.

The submaxillary gland virus as it occurs in rodents is not very virulent, and it is only by intracerebral inoculation of large doses that it produces any symptoms which we can recognize. Since we have found it impossible to demonstrate a virus in human salivary glands, no statement can be made at the present time as to whether the inclusion bodies found in the lungs of children dying of pertussis are due to the activation of an endogenous virus or to invasion by a specific virus which is the cause of whooping cough.

### CONCLUSIONS

- 1. Acidophilic intranuclear inclusion bodies occur in the salivary glands of Chinese infants dying from miscellaneous causes. The lesion is similar to that previously described in infants in Europe and America.
- 2. Attempts to prove that this lesion is due to an infectious agent by its production in animals have been unsuccessful.
- 3. Acidophilic intranuclear inclusion bodies have been found in the submaxillary glands of hamsters, white mice, and wild rats.
- 4. Evidence is presented to show that the lesion in hamsters, white mice, and wild rats is due to a virus, which is specific for each species, being transmissible to normal individuals of this breed, and which is very similar to the submaxillary gland virus of guinea pigs.

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# EXPLANATION OF PLATES

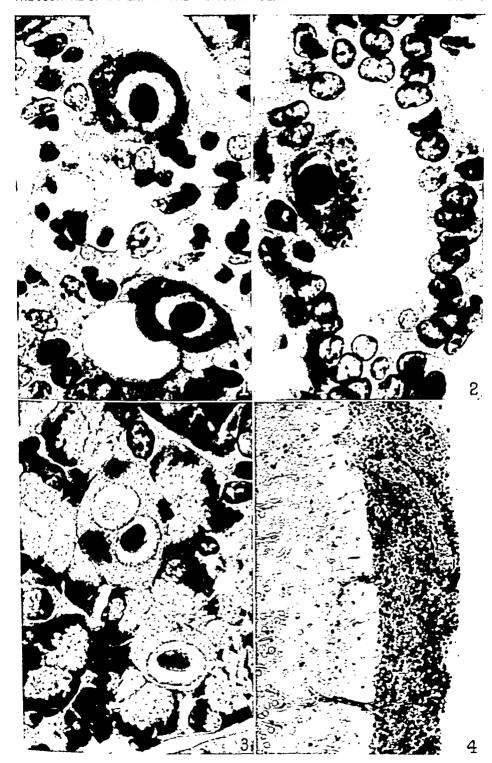
- Fig. 1. The submaxillary gland from Case 1 showing two hypertrophied duct cells with inclusion bodies surrounded by well developed halos. Eosin and methylene blue.
- Fig. 2. The submaxillary gland from Case 3 showing a greatly hypertrophied duct cell which has been extruded into the lumen. The nucleus is almost completely filled by the inclusion body and the halo is not well defined. The cytoplasm of the cell contains numerous basophilic masses.
- Fig. 3. Submaxillary gland of a full grown hamster showing hypertrophied acinal cells, two of which have fused. The nuclei are greatly enlarged and contain large inclusion bodies surrounded by halo. deeply in certain areas than in others. The cytoplasm of the cells stains more
- Fig. 4. Low power section of the brain of a young hamster inoculated with an emulsion of the submaxillary glands of full grown hamsters. A well marked meningeal exudate is shown. × 155.

- Fig. 5. High magnification of Fig. 4 showing meningeal exudate, composed of mononuclear cells, with an inclusion body surrounded by a wide halo.
  - Fig. 6. Submaxillary gland of a full grown white mouse showing a hyper-

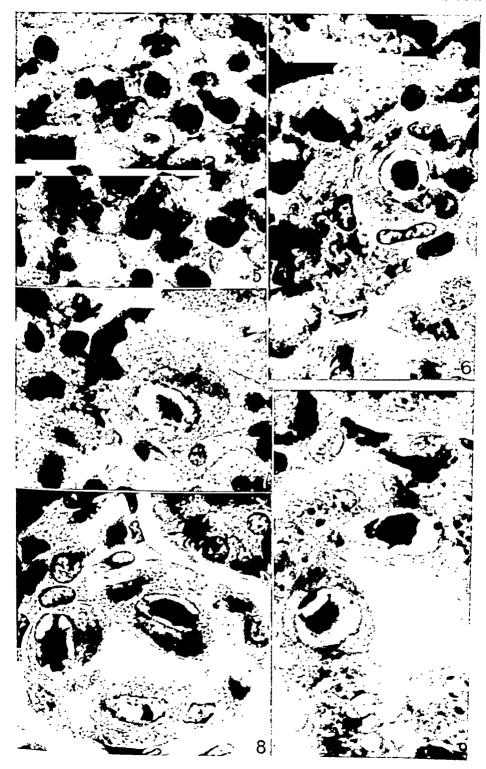
trophied acinal cell. The nucleus is greatly enlarged and contains a large inclusion body surrounded by a clear space. The nuclear chromatin is collected in several small masses at the surface of the nuclear membrane.  $\times$  1290.

- Fig. 7. Submaxillary gland of a wild rat showing a hypertrophied acinal cell. The nucleus is enlarged and shows an inclusion body surrounded by a wide halo.
- Fig. 8. Submaxillary gland of a wild rat showing two hypertrophied duct cells. Both cells contain large, irregular inclusion bodies. × 1290.
- Fig. 9. Submaxillary gland of a guinea pig showing hypertrophied duct cells with inclusion bodies, for comparison with Fig. 8.  $\times$  1290.





(Kuttner and Wang, Inclusion bodies in salivary plands)



(Kuttner and Wang: Inclusion bodies in salivary glands)

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